# EXHIBIT 7

# Solid-Phase Supports for Oligonucleotide Synthesis

**UNIT 3.1** 

#### INTRODUCTION TO SOLID-PHASE SYNTHESIS

The quest to understand and create biological molecules has long challenged synthetic chemists. In particular, the chemical synthesis of peptides and nucleic acids has always been a major pursuit. The primary structure of these molecules is a linear assembly of repeating units linked together in a defined orientation. Although solution-phase synthetic methods for coupling small units together were developed many years ago, the large number of couplings needed to assemble useful sequences was daunting. This was because each step required some type of workup, extraction, or purification, and the labor and cumulative loss of material from all the manipulations rapidly became significant problems. Indeed, the pioneering work by Khorana (1979) on total gene synthesis was not considered of practical importance by some researchers because of the enormous effort involved.

The problems involved in performing so many repetitive steps were addressed by Merrifield (1965) with the introduction of solidphase synthesis (Fig. 3.1.1). In this strategy, a large insoluble support is covalently linked to the end of the sequence being assembled. The product on the surface of the support is available to react with reagents in the surrounding solution phase. The extended products remain covalently linked to the insoluble support while unreacted reagents remain free in solution. Therefore, at the completion of each step, the products can be rapidly and conveniently isolated by simply washing the unbound reagents away from the support. This can be performed as easily as filtering off the support and washing it with solvent. The support with its attached product is then ready for immediate use in the next step, as long as moisture contamination has not been introduced (in which case the support must be dried before use). In practice, it is convenient to handle the supports inside sealed reactors or columns so exposure to the atmosphere is minimized. This is also ideal for automation and the necessary reagent additions and solvent washes are readily mechanized. The process of adding each unit is repeated over and over until the desired sequence has been assembled on the surface of the support. The product can then be released from the support by cleavage of the covalent attachment (linker arm), and after removing the protecting groups, the synthesis is complete.

This strategy was originally applied to peptide synthesis, but it is also applicable to other linear macromolecules, such as DNA and RNA (Beaucage and Iyer, 1992) and oligosaccharides (Adinolfi et al., 1996). Recently, there has been a great deal of interest in applying this strategy to the combinatorial synthesis of small molecules and a new field of solid-phase organic chemistry (SPOC) is rapidly developing (Fruchtel and Jung, 1996; Porco et al., 1997). In this review, the main focus is on supports for oligodeoxyribonucleotide and oligoribonucleotide synthesis. The synthetic strategies are often similar, particularly when synthetic libraries are prepared.

## ADVANTAGES OF SOLID-PHASE SYNTHESIS

The principal advantage of solid-phase synthesis is the ease with which immobilized products can be separated from other reactants and by-products. The simple filtration and washing steps are readily automated, and the method is ideal for the synthesis of linear molecules, which require the repetition of the same steps for every chain extension cycle. The use of insoluble solid-phase supports also permits relatively small quantities of material to be synthesized, because the additional physical bulk of the support, which is ~10 to 100 times the mass of the attached nucleoside, can be handled more easily than the nucleoside alone. Also confinement of the support inside a synthesis column eliminates handling losses. A small synthesis scale is important because of the high cost of reagents. Very little material is required for many biochemical applications and most syntheses actually prepare much more material than required. Therefore, as instrumentation has improved, the synthesis scale has decreased. Presently, synthesis on a 40-nmol scale, instead of a 0.2- to 1-µmol scale, is preferred for may applications. Oligonucleotide synthesis on a picomole scale or less may eventually become more common (Weiler and Hoheisel, 1997). It is already possible to synthesize molecules on single beads and to characterize the picomole quantities of synthetic peptides (Rapp, 1997) or oligonu-

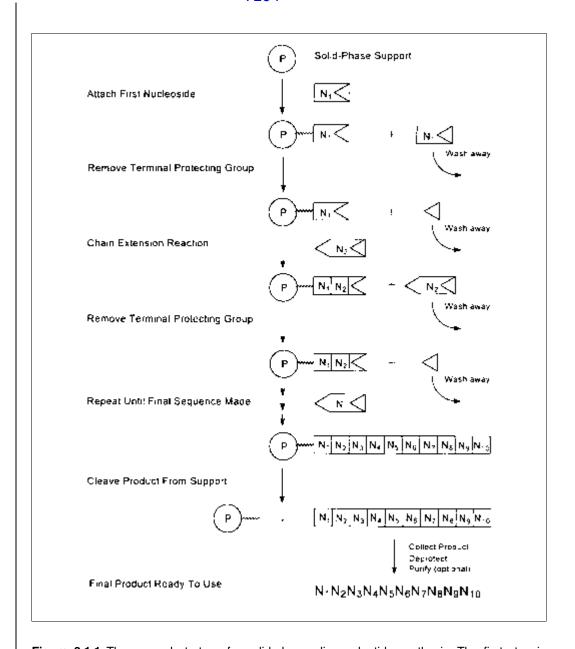


Figure 3.1.1 The general strategy for solid-phase oligonucleotide synthesis. The first step is attachment of a mononucleoside/tide (N1) to the surface of an insoluble support (P) through a covalent bond. Excess monomers, which are not chemically attached to the support, are washed away. Before chain elongation can proceed, the terminal-protecting group (◁) on the nucleoside must be removed. This exposes a free 5'-OH or 3'-OH group, depending on the orientation of the synthesis. Usually synthesis proceeds from the 3'- to 5'-direction and the terminal protecting group is an acid-labile DMTr group. The next nucleotide unit  $(N_2)$  can then be added using the appropriate synthesis chemistry (usually phosphoramidite). An excess of reagent is used to force the coupling reaction to occur on as many of the immobilized nucleotides as possible. After the coupling reaction, excess reagents are washed away. Depending on the coupling chemistry, the reaction is followed by a capping step, to block off nonextended sites, and an oxidation step (these steps are not shown; see UNIT 3.3 for details) to complete the chain-extension cycle. The process of terminal-protecting group removal and chain extension is then repeated, using different bases, until the desired sequence has been assembled. Some or all of the protecting groups may optionally be removed, and then the covalent attachment to the support is hydrolyzed to release the product. After removal of any remaining protecting groups, the oligonucleotide is ready for purification and use.

cleotides (Seliger et al., 1997) present on single beads.

The simplicity and similarity of the steps required for each chain extension reaction also greatly facilitate synthesis of modified oligonucleotides. As long as the modified substituents do not require any incompatible chemical treatments (i.e., to remove protecting groups), the inclusion of different bases and nucleosides, linkage inversions, branch points, non-nucleotide units, and end modifications can be readily accomplished. This is particularly so when the modified substituents are available as phosphoramidite derivatives, which use the same coupling chemistry as do regular bases (Beaucage and Iyer, 1993). Chimeric oligonucleotides containing peptide or peptide nucleic acid (PNA) sequences can, however, also be prepared (Bergmann and Bannwarth, 1995; Hyrup and Nielsen, 1996; van der Laan et al., 1997). Although, in these cases, the different coupling conditions and protecting groups require much more attention to ensure overall compatibility.

Finally, combinatorial methods can be used to create large numbers of different sequences. In the simplest application, multiple bases ("mixed bases") can be incorporated at defined positions by using a mixture of different monomers, instead of a single monomer, in the chain extension reaction. This procedure was originally developed to prepare oligonucleotide probes from peptide sequences when the exact codon usage was unknown. Later, this method became important when large libraries of degenerate or random sequences were required for in vitro selection experiments, such as the systematic evolution of ligands by exponential enrichment (SELEX) technique (Gold et al., 1995; see Chapter 9). Although DNA synthesizers can prepare mixed-base sites by on-line mixing, large numbers of degenerate sites are best made up by manually preparing solutions containing the desired ratio of nucleotides and incorporating the premixed reagents on the synthesizer. This is also the procedure used in base doping, when only one base, at random, within a particular section needs to be mutated (Hermes et al., 1989).

Another combinatorial approach was developed to simplify the synthesis of large numbers of oligonucleotides. This procedure used cellulose disks of filter paper as the insoluble support and became known as filter disk or segmented solid-phase synthesis (Frank et al., 1983; Matthes et al., 1984; Ott and Eckstein, 1984; Frank, 1993). In this procedure, multiple filter disks

(each producing one unique oligonucleotide) are stacked together and handled at once. Reagents can be easily passed through the stack from top to bottom, and the number of oligonucleotides synthesized is limited only by the maximum stack height that can be manipulated. A different oligonucleotide sequence is prepared on each disk by interrupting the synthesis after each chain extension step. The individual filter disks are then sorted into separate piles according to the next base to be added. The insoluble support in this case provides the means to sort the products and to separate them from the excess reagents. For normal oligonucleotides, the sorting results in a maximum of four piles, because only dA, dC, dG, and T base additions are required. Thus an operator manipulating four concurrent syntheses can produce a large number of oligonucleotides per day. This method is not limited to paper filter disks; stackable "synthesis wafers" containing packets of support in bead form have also been used. The sorting step, however, is quite difficult to automate; and although semimechanized instruments have been reported (Seliger et al., 1987; Beattie et al., 1988), the segmented approach has not been widely adopted.

The ease with which immobilized oligonucleotides can be manipulated has also lead to the development of combinatorial strategies for the synthesis of oligonucleotide libraries. Unlike the above strategies, which release the oligonucleotide product from the support at the end of the synthesis, the oligonucleotides are left attached to the insoluble support (Markiewicz et al., 1994). This method can be used to create dispersed libraries, when the sequences are prepared on separate beads, or integrated libraries, when one- or two-dimensional arrays of sequences are prepared on a single surface. The sequence identity of each element in an integrated array is known from its spatial coordinates, whereas the sequence of elements in a dispersed library must be deduced from either direct sequencing (Seliger et al., 1997) or other sequence tags. The most elegant and powerful demonstration of this technique is the synthesis of high-density arrays on small (1.28 cm<sup>2</sup>) glass chips using photolithography and light-sensitive protecting groups (Fodor et al., 1991). With the appropriate masking, any set of oligonucleotides of length N can be performed using only 4N coupling steps, and this technique can produce arrays of >10<sup>6</sup> different sequences (Lipshutz et al., 1995; McGall et al., 1996). Other combinatorial strategies using either glass plates (Milner et al., 1997) or

7106

polypropylene sheets (Matson et al., 1995; Weiler and Hoheisel, 1996) as the insoluble support have been described for the synthesis of oligonucleotide arrays, although the array densities were much lower.

#### DISADVANTAGES OF SOLID-PHASE SUPPORTS

Although a powerful technique, solid-phase synthesis has some drawbacks. The main limitation is the need for very high coupling yields in every chain extension step. This is because the overall yield of product decreases rapidly as the number of consecutive chain extension steps increases (Fig. 3.1.2). For example, if each base addition step had a yield of 90%, then the amount of dinucleotide produced (one base addition) is 90%. The yield of trinucleotide (two base additions) is  $0.90 \times 0.90 \times 100\% =$ 81%; the yield of tetranucleotide (three base additions) is  $0.90 \times 0.90 \times 0.90 \times 100\% = 73\%$ ; and so on. Note that the first nucleoside is attached to the insoluble support before the start of oligonucleotide synthesis and the efficiency of that step is not included in the calculation. The mathematical relationship between the overall yield (OY) and the average coupling efficiency (AY) is either

$$OY = \left(\frac{AY}{100}\right)^n \times 100\%$$

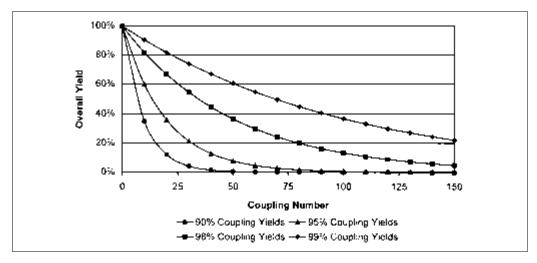
or

$$OY = \left(\frac{AY}{100}\right)^{N-1} \times 100\%$$

where n is the number of coupling steps and N is the length of the oligonucleotide. The second equation assumes that the synthesis was performed by extending the product by one base at a time, as is usual.

The consequence of the exponential relationship between overall yield and average coupling efficiency is that long oligonucleotides cannot be prepared without very high yields in every step. The most difficult step is usually the coupling reaction; but in some strategies (e.g., light-directed synthesis of arrays or the use of liquid-phase supports), quantitative removal of the terminal-protecting group is also problematic. Coupling yields that would be acceptable for most solution-phase reactions (e.g., the 90% yield assumed in the above example) are not adequate; only yields >98% are acceptable. The lack of a coupling reaction that could reliably produce such high efficiencies was the major reason why solid-phase oligonucleotide synthesis was not successful until the early 1980s. After the discovery of trivalent phosphite-coupling chemistry and phosphoramidite derivatives (Caruthers, 1991), however, average coupling efficiencies of 99% or more were possible. Such high coupling efficiencies now allow oligonucleotides as long as 200 bases to be prepared (Bader et al., 1997b).

Another consequence of producing less than 100% coupling efficiencies is the accumulation of failure sequences containing deletions. The number of these failure products can be greatly reduced by the addition of a capping step after each chain extension reaction. This step, which typically uses acetic anhydride to acetylate nonextended molecules, prevents the failure



**Figure 3.1.2** Overall yield vs. number of couplings. The overall yield of full-length product decreases with the number of coupling reactions for average coupling efficiencies of 90%, 95%, 98%, and 99%.

sequences from participating in any further reactions; however, a series of failure sequences, each one base shorter than the desired full-length product, will be present at the end of the synthesis.

Separating the full-length product (of length N) from the shorter failure sequences and especially the N-1 failure sequence is another significant problem. This purification step becomes more difficult as oligonucleotide length increases, and for oligonucleotides greater than ~30 bases long, only polyacrylamide gel electrophoresis (PAGE) has sufficient resolving power to separate the full-length product from the N-1 component. Fortunately, however, many biochemical applications do not have stringent purity requirements; and if the coupling efficiency was high enough, the mixture of products produced can often be used with either minimal (desalting) or no purification (Pon et al., 1996).

Analysis of the synthetic products still attached to the surface of the insoluble support also presents a major difficulty for researchers developing new techniques or new solid-phase supports. This is an especially significant problem for applications using immobilized arrays, because removal of the products for characterization is often difficult, if not impossible. Nuclear magnetic resonance (NMR) studies of immobilized products on solvent-swollen (gelphase) polymers (Bardella et al., 1993) can be performed; but because such supports are not preferred for oligonucleotide synthesis, there have been few studies relating to oligonucleotide synthesis. Rigid supports can be studied using NMR and magic angle spinning, but there has been only one report of <sup>31</sup>P NMR performed on controlled-pore glass (CPG) particles with oligonucleotides (Macdonald et al., 1996). Recently, ellipsometry, interferometry, and optical wave guides have been used to study oligonucleotide arrays (Stimpson et al., 1995; Gray et al., 1997), but these techniques do not provide specific information about the fidelity of the oligonucleotide synthesis.

Finally, the cost of the support is a major factor when performing large-scale syntheses because, even with high loading supports, ~3 g of support is required for each gram of oligonucleotide product. Because most supports are expensive and can be used only once, there is a strong economic incentive to develop methods for regenerating and reusing the supports, especially when tonne quantities of products are required. Recently, examples of up to 12 syntheses of oligonucleotide phosphodiester

(Pon et al., 1999) and phosphorothioate (Pon et al., 1998) sequences on the same reusable supports have appeared, and further improvements in this area are expected.

### CHEMICAL REQUIREMENTS FOR SOLID-PHASE SUPPORTS

A wide variety of different insoluble support materials have been developed for different applications. The ideal support should contain an appropriate chemical group on its surface that can be selectively coupled, usually through a linker arm, to the first monomer unit. Normally, supports for oligonucleotide synthesis are purchased with primary amino group functionality, although hydroxyl and carboxyl derivatized supports may also be obtained. The amount of surface derivatization (loading) on the support determines the maximum amount of product that can be prepared. Supports with loadings of 100 to 1000 µmol/g or more are available for the synthesis of either peptides or small molecules (Winter, 1996). Oligonucleotide synthesis, however, is almost always performed using nucleoside loadings of less than ~100 µmol/g; optimum results are obtained on supports with less than ~40 µmol/g of nucleoside, because the efficiency of coupling decreases as the number of molecules on the surface increases. Because the amount of support can usually be increased to accommodate the scale required, supports with loadings >50 µmol/g are not commonly used. The lower coupling efficiencies obtained with higher loaded supports can actually make it counterproductive to use these materials in most automated DNA synthesizers.

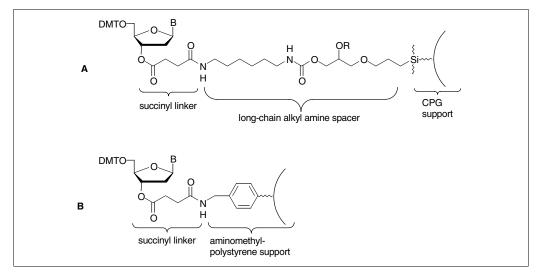
The structure of the compound(s) used to join the surface of the support to the first nucleoside is also of critical importance (Fig. 3.1.3). This attachment is generally composed of two distinct portions. The first portion is the spacer that connects the active functional group (usually NH<sub>2</sub> or OH groups) to the matrix of the insoluble support. This spacer can be as simple as a single methylene group (e.g., aminomethyl polystyrene) or it can be a lengthy alkyl or alkyloxy chain (e.g., long-chain alkylamine CPG). Generally, a long chain is preferred to distance the terminal functional group from the support's surface. Usually, the supports are sold with a satisfactory spacer, but sometimes additional spacers are added to change the terminal functional group or to increase the overall length (Katzhendler et al., 1987; van Aerschot et al., 1988; Arnold et al., 1989).

A second difunctional molecule is then required to connect the amino group on the support to the first nucleoside unit. In oligonucleotide synthesis, this structure is commonly referred to as a linker or linker arm. In peptide synthesis and combinatorial synthesis, this structure is referred to as either a handle or anchor, terms not usually associated with oligonucleotide synthesis. In oligonucleotide synthesis, the linker arms are usually dicarboxylic acids, such as succinic acid (Pon et al., 1988; Damha et al., 1990; Bhongle and Tang, 1995) or hydroquinone-O,O-diacetic acid (Pon and Yu, 1997a), that connect the nucleoside to the support via ester and amide bonds. The length, rigidity, and hydrophobicity of the linker arm can affect coupling efficiency (Katzhendler et al., 1989), and the chemical stability restricts the conditions that can be used during synthesis. This affects the choice of protecting groups. Most linkers for oligonucleotide synthesis are resistant to acidic conditions and cleavable by basic conditions. This allows the most popular combination of protecting groups—acid-labile 5'-dimethoxytrityl (DMTr) groups and baselabile N-acyl and cyanoethyl phosphate protecting groups—to be used. As will be discussed later, the speed with which the linker arm can be cleaved is also an important consideration. Strategies that require removal of oligonucleotide-protecting groups without cleavage from the support also require linker arms that are either very stable or removable using

conditions orthogonal to the deprotection conditions. Finally, different linker arms can be used to prepare oligonucleotides with terminal end modifications, such as 3'-phosphate, amino, carboxyl, thiol, or other substituents.

The chemical properties of the rest of the surface should be either inert or capable of being made inert by silvlation, benzovlation, or other similar passivating treatment (Pon, 1993; Tang and Tang, 1997). This is because residual groups, such as amino, hydroxyl, or silanol groups, can also react with phosphoramidite derivatives. This creates failure sequences coupled to the support through either phosphoramidate or phosphodiester linkages and lacking the correct 3'-nucleoside. When coupling reactions are monitored by quantitation of the orange dimethoxytrityl cation released during detritylation, the formation of such failure sequences can be deduced from apparent coupling yields of >100%. Fortunately, the phosphodiester linkages to the surface of the support are difficult to hydrolyze, and these failure sequences are not released from the support by the usual cleavage conditions (Pon et al., 1988).

The hydrophobicity of the support's surface is another consideration. Supports that are quite hydrophobic, such as polystyrene and benzoylated polymethacrylate, are sometimes preferred over supports such as CPG and polyethylene glycol (PEG), which have hydrophilic surfaces (McCollum and Andrus, 1991; Tang



**Figure 3.1.3** The structure of the two most commonly used solid-phase supports for oligonucleotide synthesis. (**A**) LCAA-CPG. (**B**) Aminomethyl polystyrene (nonswelling and highly cross-linked). In both cases, a nucleoside is attached to the amino group of the support through a succinic acid linker, which can be cleaved by ammonium hydroxide after the synthesis. DMT, 5′-dimethoxytrityl (DMTr).

and Tang, 1997). All phosphoramidite-coupling reactions are sensitive to moisture contamination, and such contamination is presumed to be more easily washed off the hydrophobic supports. This may allow greater synthesis efficiency with smaller excesses of reagent; however, quantitative comparisons are difficult to make, and both rigid polystyrene and CPG supports are widely used.

## PHYSICAL AND CHEMICAL PROPERTIES OF SOLID-PHASE SUPPORTS

The accessibility of the support's surface to incoming reagents is probably the most important consideration when choosing the physical properties of the insoluble support. Although a greater surface area provides higher capacity, increased porosity must be balanced against steric restrictions and rate-limiting diffusion. The following sections discuss the major classes of solid-phase supports, categorized by the type of surface accessibility.

#### **Liquid-Phase Supports**

Liquid-phase supports are high molecular weight polymers that can be completely dissolved in the solvents required for synthesis but can be precipitated or crystallized in other solvents or solvent conditions. When they are dissolved in solution, coupling reactions on liquid-phase supports are performed in the same manner as conventional solution-phase synthesis. After completion of the coupling reaction, however, the liquid-phase support is precipitated by adding a solvent in which it is insoluble. The resulting precipitated support can then be filtered off and washed free of excess reagents in the same manner as other insoluble supports. After the washing step, the support is redissolved in the appropriate solvent, and the synthesis continued. Alternately, dialysis or ultrafiltration can also be used to remove low molecular weight impurities. The most widely used liquid-phase supports are PEG polymers (Bonora, 1995) with average molecular weights varying between 5,000 and 20,000, although cellulose acetate (Kamaike et al., 1988) and poly(*N*-acryloylmorpholine) polymers (Bonora et al., 1996) have also been used. These supports are soluble in solvents such as dichloromethane, pyridine, and acetonitrile but insoluble in solvents such as ethers and alcohols. Nucleoside loadings of ~100 to 200 µmol/g are generally obtained, and the purified yield of oligonucleotide 8- to 20-mers is about 100 mg per gram of starting support (Bonora, 1995).

The advantages of having a homogeneous solution include lower costs, because less reagent excess is required, and the ability to use spectroscopic methods (UV/VIS, NMR, Fourier transform-IR) to monitor the reactions and quality of the immobilized products. Furthermore, the method does not require any elaborate instrumentation because the reactions and precipitation/filtration steps are performed in ordinary glassware. Consequently, liquid-phase supports were some of the earliest supports to be used (Cramer et al., 1966; Hayatsu and Khorana, 1966). The method, however, is not easily automated, and each chain extension cycle requires several hours to perform. Moisture contamination must also be scrupulously avoided, because the PEG supports are very hydrophilic. Nevertheless, this method is suitable for larger scale oligonucleotide synthesis, when cost is more important than speed.

#### **Gelatinous Polymer Supports**

The first insoluble supports developed were polystyrene-divinylbenzene polymers with only a small amount (1% to 5%) of cross-linking (Pon, 1993). These supports could swell up to five times their dry volume in nonpolar solvents, such as dichloromethane, to provide a large surface area and loading capacity (0.1 to 1.5 mmol/g). Other swellable polyacrylamide-containing supports, with loadings up to 5 mmol/g, have also been developed (Winter, 1996). In these supports, up to 99% of the reactive sites are located inside the bead. They are classified as gelatinous polymer supports because of the gel-like environment in which the reactions take place (Rapp, 1996). These supports perform very poorly when used for solid-phase oligonucleotide synthesis because they don't swell satisfactorily in the polar solvents required and because reagent diffusion into and out of the supports is slow. Therefore, the swollen beads can be used only in batch reactors and not in continuous flow synthesizers (Belagaje and Brush, 1982; Ito et al., 1982; Ohsima et al., 1984). Only the very earliest oligonucleotide syntheses were attempted on these types of supports; and with one exception (Montserrat et al., 1994), their present use is restricted to peptide synthesis.

#### **Macroporous Supports**

The difficulties mentioned above were overcome by the development of rigid macroporous

7110

supports. These supports are based on inorganic materials, such as silica gel and CPG, or highly cross-linked polymers, such as polystyrene or polymethacrylate. Well-defined pores are created in these supports to increase the surface area and loading capacity. They do not become swollen with solvent and have permanent porosity. Their rigidity allows them to be used in packed continuous-flow columns, and their properties are very similar to the packing materials used in HPLC separations. The maximum loading possible on the rigid supports, however, is much less than on the swellable supports.

Silica gel and porous glass supports are ideal nonswelling materials that are readily derivatized using techniques developed by the glass fiber and chromatography industries. CPG beads, which are stronger and easier to handle than is silica gel, are preferred and are available in three particle sizes—125 to 177 µm, 74 to 125  $\mu$ m, and 37 to 74  $\mu$ m—with large (75 to 4000 Å in diameter) and very uniformly sized pores. The maximum pore diameter distribution is only  $\pm 10\%$  for 80% of the pore volume. A variety of chemically derivatized CPG supports with different functional groups is available, including magnetic CPG beads. The surface area and loading depend on the pore size; the beads with larger pores have lower loadings.

Long-chain alkylamine (LCAA) derivatized CPG supports with 500-Å pores and amino loadings of ~100 µmol/g are the most commonly used. These supports are usually derivatized with 30 to 40 µmol/g of nucleoside (Pon, 1993) and are suitable for the synthesis of oligonucleotides of up to 50 to 60 bases. The 500-Å pore size begins to restrict the coupling efficiency of longer oligonucleotides because of steric factors; however, much longer oligonucleotides (100 to 150 bases) can be prepared on 1000-Å CPG supports (Efcavitch et al., 1986). Synthesis of very long oligonucleotides also benefits from a support with a low surface loading (~5 µmol/g), because it contributes to greater coupling efficiency.

Highly cross-linked rigid polymer beads with large pores have also been developed as an alternative to CPG. These were developed primarily to overcome cost and supply problems associated with CPG supports. The greater inertness of the polymers relative to CPG, especially during alkaline deprotection conditions, was also an advantage.

The first rigid synthetic polymer, introduced by Perkin-Elmer/Applied Biosystems Division (PE/ABD), was a highly cross-linked polystyrene support with 1000-Å pores (McCollum and Andrus, 1991). These supports produce excellent quality oligonucleotides, and prepacked ABI LV40 (40 nmol) and ABI LV200 (200 nmol) columns are widely used. The nucleoside capacity of the supports, however, is lower than that of CPG, and prepacked columns >200 nmol are not available.

A second rigid polymer based on a polymethacrylate vinyl alcohol copolymer with 1000-Å pores has also been used (Reddy et al., 1994b). This copolymer is sold as a chromatographic medium by both Merck and Toso-Haas, respectively, under the trade names Fractogel and Toyopearl. It can be purchased with either hydroxyl functional groups (for size exclusion chromatography) or amino functional groups (for affinity chromatography). These supports are easy to handle, durable, and inexpensive, and their loading capacity is much higher (up to 135 μmol of nucleoside/g) than either rigid polystyrene or CPG supports.

#### Composite macroporous supports

Composite supports have been prepared that combine the advantages of gelatinous and rigid supports. These supports are prepared by polymerizing a low cross-linked polyacrylamide inside the pores of a rigid macroporous substrate, such as silica gel, or highly cross-linked polystyrene beads. Typical capacities are between 100 and 500 µmol/g. The soft gelatinous phase is protected by the rigid carrier, and these supports can be used in a continuous flow column system. The supports, however, are fragile, and swelling differences can create unwanted fines. Although this type of support was once used for oligonucleotide synthesis by the phosphotriester method (Gait et al., 1982), present use of commercially available composite supports is limited to other fields.

#### **Grafted Polymeric Carriers**

Another method of creating hybrid supports, which combine the advantages of gelatinous supports and rigid supports, is to covalently couple or graft long polymeric chains onto the surface of a rigid support. The surface polymers are not cross-linked and are readily solvated, whereas the rigid core remains insoluble. The low thickness of the surface layer and the absence of cross-linking increase the rate of mass exchange and allow a large number of functional sites to be introduced for nucleoside attachment (up to 160 µmol/g). These supports can, therefore, be used in continuous flow column synthesis because the supports are me-

chanically stable, do not show significant swelling, and allow reagents to be removed using short wash steps.

The first example of this type of support in oligonucleotide synthesis (by the phosphodiester method) was a polystyrene-polytetrafluoroethylene (PS-PTFE) graft copolymer. This was prepared by <sup>60</sup>Co irradiation of PTFE beads and vapor-phase styrene deposition (Potapov et al., 1979). Another coated Teflon support, in the form of fibers not beads, was also commercially available in the 1980s (Bower et al., 1987; Duncan and Cavalier, 1988). More recently, PS-PTFE beads have been found very satisfactory for oligonucleotide synthesis using the phosphoramidite method (Birch-Hirschfield et al., 1996).

Another group of widely used graft copolymers are the polyethylene glycol-polystyrene (PEG-PS) tentacle polymers produced by Rapp Polymere under the TentaGel trade name (Rapp, 1996). These supports are prepared by anionic polymerization of ethylene glycol on hydroxyl derivatized cross-linked polystyrene. Copolymers with PEG chains of about 3000 Da are considered optimal, but the polymerization process can produce PEG chains as large as 20,000 Da. Unlike the previous hydrophobic PS-PTFE copolymers, the PEG-PS copolymers have an insoluble polystyrene core and a hydrophilic PEG coating. The relative amounts of material in the coating and the core are also quite different. The PS-PTFE supports have between 2% and 10% polystyrene as the surface coating, whereas the PEG-PS supports have 70% PEG as the coating and only 30% polystyrene as the core. Therefore, the properties of the TentaGel resins are mostly dictated by the PEG coating. The supports swell considerably (3 to 5 times dry volume) in solvents that dissolve PEG, but owing to the insoluble core, they are suitable for both batch and continuous flow processing. The gel-like environment surrounding these supports allows coupling reactions to proceed in a manner similar to solution-phase reactions. This environment presumably allows cyclization reactions to proceed much more efficiently than on CPG supports, and circular oligonucleotides of up to 32 bases have been prepared (Alazzouzi et al., 1997). A number of different TentaGel resins are commercially available with different functional end groups, particle sizes (ranging from uniformly sized 10 µm beads to 750 µm macrobeads), and loading capacities (0.25 to 1.3 mmol/g) for all types of solid-phase and combinatorial synthesis (Winter, 1996). The high capacity of these supports has found particular use in large-scale (200 to  $1000\,\mu\text{mol}$ ) oligonucleotide synthesis (Wright et al., 1993).

#### **Nonporous Supports**

Rigid nonporous beads without surface copolymerization have also been used as supports of oligonucleotide synthesis, although the capacity is two to three orders of magnitude less than similar porous supports. Nonporous silica beads allow long oligonucleotides to be prepared with high coupling yields because of the absence of restrictive pores (Seliger et al., 1989, 1995). The very small diameter (1.5  $\mu$ m) particles required to provide an acceptable surface loading (2  $\mu$ mol/g), however, made this support very difficult to work with. Similar handling problems also occurred when nonporous 4.5- $\mu$ m magnetic Dynabeads were used (Albretsen et al., 1990).

A more practical application for nonporous supports is the synthesis of immobilized oligonucleotides. Although a large number of methods have been developed to immobilize previously synthesized oligonucleotides on insoluble supports, it is simpler to synthesize the oligonucleotide directly onto the support required for the final hybridization assay. This type of synthesis requires a stable linker that can withstand the conditions used to remove all of the protecting groups (typically 55°C) NH<sub>4</sub>OH, 16 hr) after completion of the synthesis. The deprotected oligonucleotides left attached to the support can then be used as hybridization probes. Both nonporous glass (Maskos and Southern, 1992) and polystyrene beads, with respective loadings of 50 to 70 and 150 nmol/g, have been used. In the latter case, time-resolved fluorescence detection on single Dynosphere beads was performed (Hakala et al., 1997).

Hybridization assays using two-dimensional formats, however, are much more common than assays using beads. Consequently, a great deal of effort has gone into the synthesis of oligonucleotide arrays on flat glass and polypropylene supports. The surfaces of glass slides can be derivatized using the same techniques developed for silica gel and CPG supports. Typical surface loadings of 10 to 40 pmol/cm<sup>2</sup> are obtained (Maskos and Southern, 1992; McGall et al., 1997), although one account of ~166 pmol/cm<sup>2</sup> was reported when phosphoramidite reagents were reacted directly with surface silanol groups (Cohen et al., 1997). Oligonucleotide synthesized with permanent linkages to quartz fibers has also been used as a DNA sensor (Uddin et al., 1997).

Recently, polypropylene sheets have been used as solid-phase supports for oligonucleotide synthesis (Matson et al., 1994, 1995). Wehnert et al., 1994). Polypropylene has the advantages of greater flexibility, physical strength, and chemical stability at high pH. It also has low nonspecific adsorption of biomolecules. One report, however, has mentioned an incompatibility between polypropylene supports and the tetrahydrofuran (THF) solvent commonly used in capping and oxidation reagents (Weiler and Hoheisel, 1996).

The inertness of this polymer makes chemical derivatization difficult. Amino-modified supports are prepared by exposure of the polypropylene surfaces to ammonia vapor inside a radio frequency plasma (RFP) generator (Chu et al., 1992). This results in an amino group loading of about 15 to 25 nmol/cm<sup>2</sup>. Oligonucleotides have been attached to these amino groups, either directly via phosphoramidate linkages (Matson et al., 1994) or through intermediate spacers with terminal hydroxyl (Shchepinov et al., 1997) or amino groups (Weiler and Hoheisel, 1996) to give supports with respective loadings of 10, 0.3, and 0.03 to 0.09 nmol/cm<sup>2</sup>. Efficient hybridization requires optimization of both loading density and spacer length, because duplex formation can be inhibited by too close spacing and by spacers that are either too short or too long.

Polypropylene can also be chemically derivatized without requiring an RFP generator. Bromination using N-bromosuccinimide and 2,2'-azobisisobutyronitrile followed by amination with long-chain diamines or amino alcohols has recently been described, but no surface loadings were reported (Seliger et al., 1995). Oxidation of polypropylene with chromium(VI) oxide followed by borane-tetrahydrofuran complex and H<sub>2</sub>O<sub>2</sub>/NaOH treatment has also been used to produce hydroxyl-derivatized polypropylene tapes. Direct reaction of phosphoramidites to this tape yielded a nucleotide loading of 7 nmol/cm<sup>2</sup>. These polypropylene tapes have been used to prepare a 200-base-long polythymidylic acid sequence and overlapping one-dimensional arrays (Bader et al., 1997a,b).

### Filter Disks, Membranes, and Sintered Blocks

This section deals with supports whose physical properties are not easily categorized. Paper filter disks probably represent the cheapest and most readily accessible insoluble support for oligonucleotide synthesis. These supports contain cellulose fibers that have many hydroxyl groups available for oligonucleotide attachment and that are resistant to all of the chemical conditions required for oligonucleotide synthesis. Paper filter disks were ideal supports for synthesizing multiple oligonucleotides simultaneously using the "segmental solid-phase" procedure, because they could be easily labeled and sorted (Frank et al., 1983; Matthes et al., 1984; Ott and Eckstein, 1984; Frank, 1993).

Another important innovation was the development of commercially available MemSyn DNA synthesis supports (Perseptive Biosystems). These contain a membrane-based support made of porous PTFE and coated with an aminopropyl linker. These membranes are sealed inside specially designed low dead volume disposable housings, which resemble common syringe filters, and have loadings of either 50 or 200 nmol. The membranes are easier to handle than are particulate supports, and mass-produced synthesis cartridges are presumably more reliable and easier to manufacture. The large pore diameter of these filter membranes (0.2 µm or 2000 Å) allow both large and small oligonucleotides to be synthesized.

Finally a new process, developed at NASA, has been used to derivatize polyethylene (Devivar et al., 1999). In this process, gaseous amine radicals are used to aminate porous polyethylene sintered blocks (FlowGenix, Webster, TX). The amino functionalized sites can then be derivatized with nucleosides for oligonucleotide synthesis. This technology allows supports in the form of plugs and disks to be produced, which should be more convenient to handle than are particulate supports.

This discussion clearly indicates that a wide range of insoluble supports have been developed for an increasing number of different applications. Selection of an appropriate support requires consideration of both the chemical properties and the physical characteristics of the support. The size and shape of support beads can vary from small uniformly sized or irregularly sized particles to large macrobeads. Supports in sheet and plate form are amenable to extremely sensitive isotopic or fluorescent detection schemes. High-density arrays or "gene chips" are also emerging as important tools for gene expression studies. Supports in membrane or foam formats provide the synthesis capacity of porous beads but greatly simplify the handling and manufacturing steps required to mass produce ready-to-use synthesis cartridges.

#### SUPPORT DERIVATIZATION: NUCLEOSIDE AND LINKER ARM COUPLING STRATEGIES

Nucleosides are attached to the surface of the support through an intermediate linker arm, whose design must be carefully considered. The linker arm should allow easy nucleoside attachment to the support and be compatible with all of the conditions required for synthesis. Furthermore, the linker must be designed to accommodate different cleavage and deprotection strategies. The many different types of possible linkages can be classified into four groups for strategies that require either:

- cleavage from the support with concomitant or postcleavage deprotection;
- deprotection of the immobilized products with optional postdeprotection cleavage;
- deprotection of the immobilized products with no cleavage from the support; or
- linkers that impart terminal end modifications to the oligonucleotide products.

The following section discusses how different linkers have been use in the first three strategies. The use of different linkers and insoluble supports in the synthesis of end-modified oligonucleotides will be discussed in future units.

#### **Linker Arms Cleaved after Synthesis**

#### Succinyl linker arm

The most commonly used linker arm in oligonucleotide synthesis is succinic acid (Fig. 3.1.3). This linker was used in the early 1970s and has remained very popular because of low cost and ease of incorporation (Yip and Tsou, 1971). Both succinyl dichloride (Sharma et al., 1992) and succinic anhydride have been used as starting materials, but the anhydride is greatly preferred because of its easier handling. A suitably protected 2'-deoxyribonucleoside can be succinylated at either the 5'- or 3'hydroxyl position, and the resulting 5'- or 3'-O-hemisuccinate is coupled to an amino- or hydroxyl-derivatized support. Alternatively, the support can be succinylated first and then coupled to a nucleoside (Damha et al., 1990). This method has the advantage of not requiring the synthesis of an inventory of succinylated

nucleosides. Coupling of a nucleoside to a succinylated support, however, is more difficult and usually gives lower nucleoside loadings than does attachment of a presynthesized nucleoside 3'-O-hemiester. The coupling reactions between the succinate and the nucleoside or support have usually been performed using carbodiimide coupling reagents, such as dicyclohexylcarbodiimide (Montserrat et al., 1993), 1-(3-dimethylaminopropyl)ethylcarbodiimide (Pon et al., 1988), and diisopropylcarbodiimide (Bhongle and Tang, 1995), and required coupling times between 1 and 24 hr. A faster coupling reaction—involving reaction of a nucleoside-3'-O-hemisuccinate with 2,2'dithiobis(5-nitropyridine) (DTNP) and dimethylaminopyridine (DMAP) followed by addition of triphenylphosphine (TPP) and LCAA-CPG—can reduce the coupling time to 2 to 30 min (Kumar et al., 1996). Extremely fast coupling of a nucleoside-3'-O-hemisuccinate to LCAA-CPG can be obtained using a variety of phosphonium or uronium coupling agents and DMAP. These reactions are complete in the time required to add the reagent to the support ~4 sec) and allow for the possibility of automated on-line support derivatization (Pon and Yu, 1997b).

After completion of the oligonucleotide assembly, the protected products can be cleaved from the support by hydrolysis of the succinyl linker arm with either concentrated aqueous ammonium hydroxide (1 to 2 hr) or gaseous ammonia at 10 bar pressure (15 min; Boal et al., 1996). Faster hydrolysis can be performed by including stronger reagents, such as methylamine (Reddy et al., 1994a) or sodium hydroxide (Chow and Kempe, 1997), with the ammonium hydroxide. These reagents can reduce the cleavage time to 5 min and speed up the removal of base-protecting groups. There are, however, potential problems with the modification of cytosine bases through either aminoalkylation (Macmillan and Verdine, 1991) or deamination (Debear et al., 1987) with these reagents.

Although the succinic acid linker has been widely used for a long time, the succinyl linker is unnecessarily stable for oligonucleotide synthesis. The relatively harsh conditions required to hydrolyze the succinyl linker are incompatible with a number of base-sensitive minor bases, backbone modifications, and dye labels; and the time required to cleave the succinyl linker with NH<sub>4</sub>OH is unnecessarily long (Alul et al., 1991; Avino et al., 1996; Pon and Yu, 1997a). Therefore, a number of more easily

Figure 3.1.4 Structure of labile linker arms that can be cleaved under milder conditions than a succinic acid linker. (A) *o*-Nitrobenzyl carbonate photolabile linker arm (Greenberg and Gilmore, 1994). (B) 5-Methoxy-2-nitrobenzyl carbonate photolabile linker arms (Venkatesan and Greenberg, 1996). (C) *o*-Nitrophenyl-1,3-propanediol base photolabile linker for 3'-phosphorylated oligonucleotides (Dell'Aquila et al., 1997). (D) Fluoride ion labile diisopropylsilyl linker arm (Routledge et al., 1995). (E) Fluoride ion labile disiloxyl phosphoramidite linker arm (Kwiatkowski et al., 1996). (F) Benzenesulfonylethyl linker arm cleavable with triethylamine/dioxane (Efimov et al., 1983). (G) NPE carbonate linker arm cleavable with DBU/pyridine (Eritja et al., 1991). (H) 9-Fluorenylmethyl linker cleavable with DBU (Avino et al., 1996; Brown et al., 1989). (J) Oxalyl linker, cleavable under very mild conditions (Alul et al., 1991). (K) Malonic acid linker for the synthesis of 3'-phosphorylated oligonucleotides (Guzaev and Lonnberg, 1997). (L) Diglycolic acid linker used to make 3'-TAMRA dye-labeled oligonucleotides (Mullah et al., 1998). (M) Hydroquinone-*O*,*O*'-diacetic acid (*Q*-linker), which can be used for routine oligonucleotides to improve synthesis productivity or to synthesize base-labile products (Pon and Yu, 1997a). DMT, 5'-dimethoxytrityl (DMTr).

cleavable linker arms have been investigated (Fig. 3.1.4).

#### Labile linker arms

Photolysis offers a very mild method for cleavage. Photolabile linker arms based on *o*-nitrobenzyl groups (Greenberg and Gilmore,

1994) have been used to synthesize oligonucleotides with 3'-hydroxyl (Fig. 3.1.4A and B), 3'-phosphate (Fig. 3.1.4C), and other 3'-end modifications. The photolysis can, however, cause small amounts (<3%) of thyminethymine photodimers, and alkaline or other conditions still need to be employed to remove

Figure 3.1.4 Continued

base-protecting groups. In addition, *N*-benzoyl-protected dA and dC nucleosides must also be avoided.

Very mild cleavage, under non-nucleophilic conditions and neutral pH, can be obtained through silyl- or disiloxyl-based linker arms (Fig. 3.1.4D and E), which are cleavable with fluoride ion. Triethylamine has been used to cleave a benzylsulfonylethanol linker arm to yield 3'-phosphorylated oligonucleotide blocks suitable for solution-phase coupling (Fig. 3.1.4F). An even more labile 2-(4-carboxyphenylsulfonyl)ethanol linker arm was considered unsuitable for phosphotriester synthesis but was not evaluated using phosphoramidite synthesis (Schwyzer et al., 1984). The non-nucleophilic base, 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU), can also be used to cleave the 2-(o-nitrophenyl)ethyloxycarbonyl (NPE) linker, N-[9-(hydroxymethyl)-2fluorenyl]-succinamic acid (Fmoc) linker, and phthaloyl linker arms (Fig. 3.1.4G to I). When using DBU cleavage, however, thymine and guanine modification can occur if methoxy- or cyanoethyl phosphate-protecting groups are not removed before the DBU treatment. Furthermore, oligonucleotides with terminal TT sequences are not efficiently cleaved.

All of the above linker arms were either difficult to prepare or did not offer any speed advantage. In addition, the requirement for deprotection conditions or reagents different from the simple NH<sub>4</sub>OH cleavage used in standard oligonucleotide synthesis procedures was an obstacle to the widespread adoption of any of these linker arms. More satisfactory alternatives would be other dicarboxylic acid linkers, especially if they were readily available and compatible with the derivatization and cleavage methods used for succinic acid.

The most labile dicarboxylic acid linker reported has been the oxalyl linker (Fig. 3.1.4J). This was completely cleaved by concentrated NH<sub>4</sub>OH in only a few seconds, and cleavage with a number of other milder reagents was also possible. The oxalyl linker, however, was too

labile for routine use, and oxalyl derivatized supports had to be used within a few weeks because significant spontaneous nucleoside loss occurred (Pon and Yu, 1997a). More stable linkages have been created using either malonic acid or diglycolic acid as the linker arm (Fig. 3.1.4K and L). Treatment of diglycolic acid (Pon and Yu, 1997a) and malonic acid (Guzaev and Lonnberg, 1997) linkers with room temperature concentrated NH<sub>4</sub>OH for 10 min was sufficient to respectively hydrolyze 68% and 90% of these linker arms, conditions that caused only 15% cleavage of the succinyl linker. The malonic acid linker arm was used in combination with a diethyl 2,2-bis(hydroxymethyl)malonate spacer to afford 3'phosphorylated methyl phosphotriester and methylphosphonate analogues. The diglycolic acid linker has principally been used in combination with a branching spacer, such as 2amino-1,3-propanediol, to prepare 3'-tetramethylrhodamine (TAMRA) labeled oligonucleotides, which are damaged by conventional ammonium hydroxide hydrolysis (Mullah et al., 1998). In this case t-butylamine/methanol/water (1:1:2) was used for cleavage from the support (20 to 60 min at room temperature) and subsequent base deprotection (1 to 3 hr at  $65^{\circ}$  to  $85^{\circ}$ C).

A more satisfactory replacement for succinic acid is hydroquinone-O,O'-diacetic acid, which is used to create a Q-linker arm (Fig. 3.1.4M). This linker is sufficiently stable so decomposition during room temperature storage is not a problem. The Q-linker, however, can be cleaved much faster than either the succinyl or diglycolic acid linkers (Pon and Yu, 1997a). For example, cleavage using NH<sub>4</sub>OH required only 2 to 3 min and cleavage using t-butylamine/methanol/water was performed in only 5 min, instead of the 45 min described above. Moreover, for routine use, supports derivatized with the Q-linker can be used without any modifications to either protecting groups, reagents, or synthesis procedures (other than a reduction in cleavage time). Thus the Q-linker can serve as a general replacement for the succinyl linker in the synthesis of either unmodified or base-sensitive oligonucleotides. The main advantage of the Q-linker, however, is the improved productivity that results from the decreased cleavage time. Unlike postsynthesis deprotection, which is performed off the automated synthesizer, the cleavage step is usually performed by the instrument; and subsequent runs cannot be started until the cleavage is complete. Because typical oligonucleotide syntheses are usually complete within 2 hr, waiting an additional 1 or 2 hr for cleavage of a succinyl linker represents a significant bottleneck.

Recently, the Q-linker arm has also been included in a strategy for oligonucleotide synthesis on reusable solid-phase supports (Pon et al., 1998, 1999). In this approach, an hydroxyl derivatized support is used to form ester linkages, which can be easily cleaved and regenerated for subsequent use. The mild cleavage conditions required to release the oligonucleotide reduce damage to the support's surface and reduce the time required to prepare the support for another use. This approach will be very useful in the large-scale (>1000 kg/y) manufacturing of oligonucleotide pharmaceuticals in which the support is the most expensive single consumable.

#### Universal linkers

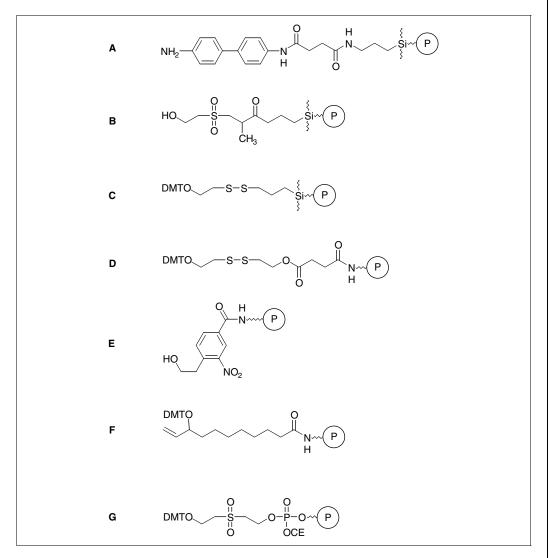
In the supports discussed above, attachment of the first nucleoside is always done separately from the actual oligonucleotide synthesis, because of the different chemistry and long coupling times required. In the past, maintaining an inventory of prederivatized supports was not problematic because of the limited number of common nucleosides. The recent development of high throughput DNA synthesizers. however, has created a need for universal supports that have the terminal nucleoside added as part of the automated synthesis. This is required not so much for inventory purposes but because manual setup of prederivatized supports is time-consuming and error prone. Universal linkers are also an advantage for the synthesis of oligonucleotides containing rare or modified bases that one wishes to incorporate at internal sites and at the 3'-end. In this way it is necessary to synthesize or purchase only the phosphoramidite derivative of the rare or modified base.

Automation of the nucleoside-coupling reaction using very fast uronium coupling reagents and DMAP is one possible approach (Pon and Yu, 1997b); however, implementation of this strategy requires construction of new DNA synthesizers with additional reagent reservoirs. A simpler approach would be to design a linker arm that could use a conventional nucleoside-3'-phosphoramidite as the first monomeric unit. It is fairly simple to design universal supports that can produce oligonucleotides with 3'-phosphate ends using either amino or hydroxyl end functions (Fig. 3.1.4A to F). Furthermore, a sulfonyldiethanol phos-

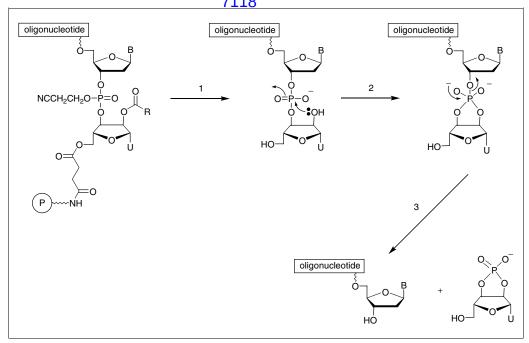
phoramidite, usually used for 5'-phosphorylation, can be used to synthesize 3'-phosphorylated sequences on any amino- or hydroxylderivatized support (Fig. 3.1.5G). Obtaining an oligonucleotide with a free 3'-hydroxyl terminus instead of a 3'-phosphate is essential, however, if the oligonucleotides are to be enzymatically extended (e.g., used as DNA sequencing or polymerase chain reaction primers). Removal of the terminal 3'-phosphate group introduced by the 3'-phosphoramidite reagents is usually difficult, because the negative charge(s) on phosphodiester and phosphomonoester bonds make them very resistant to hydrolysis. This difficulty can be overcome by introducing

a neighboring hydroxyl group so a cyclic phosphate can form via an intramolecular attack (Fig. 3.1.6). A linker containing a single ribonucleoside in an inverted orientation, so chain extension occurs from the 2′- (or 3′-) hydroxyl position and not the 5′-position, will allow cleavage of the phosphate group via a mechanism similar to the alkaline cleavage of RNA. This will produce the desired oligonucleotide with a 3′-hydroxyl terminus and a uridine-2′, 3′-cyclic phosphate.

This strategy was first used on cellulose supports (Crea and Horn, 1980; van der Marel et al., 1982); however, because dinucleotide units with inverted 3'-2' (3') linkages were



**Figure 3.1.5** Structure of universal linkers for 3'-phosphorylated oligonucleotides (see Fig. 3.1.4C and K). (**A**) Benzidine linker arm (Markiewicz and Wyrzykiewicz, 1989). (**B**) Hydroxyethylsulfonyl linker arm (Markiewicz and Wyrzykiewicz, 1989). (**C**) Hydroxyethyl disulfide linker (Kumar et al., 1991). (**D**) Hydroxyethyl disulfide linker (Asseline and Thuong, 1989; Gupta et al., 1991). (**E**) NPE linker (Eritja et al., 1991). (**F**) Universal allyl linker, 9-*O*-(4,4'-dimethoxytrityl)-10-undecenoic (Zhang and Jones, 1996). (**G**) Dimethoxytrityl sulfonyldiethanol phosphoramidite linker (Bader et al., 1997b; Shchepinov et al., 1997). DMT, 5'-dimethoxytrityl (DMTr).

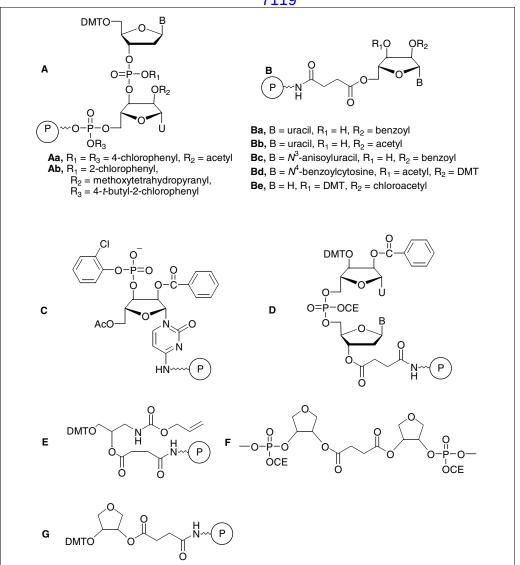


**Figure 3.1.6** Mechanism of terminal phosphate cleavage in universal supports. (1) The first step in the cleavage/deprotection process is hydrolysis of the ester from the hydroxyl group adjacent to the first phosphate linkage. This step occurs concurrently with the removal of the cyanoethyl groups on the phosphate linkages and hydrolysis of the ester attachment to the support. (2) The deprotected hydroxyl group can then cyclize by attacking the phosphorus atom. (3) Formation of a 2',3'-cyclic phosphodiester releases the oligonucleotide sequence with a free 3'-OH group.

prepared in solution before attachment to the support the method was not truly universal (Fig. 3.1.7A). The universal support concept was first fully examined when uridine mononucleosides were attached to CPG through 5'-succinate linkages (Fig. 3.1.7Ba to c). Oligonucleotide synthesis, using nucleoside-3'-phosphoramidites, can then be performed from the 2' (or 3') hydroxyl position of the uridine linker in the normal manner. Cleavage from the linker, however, involves two steps: hydrolysis of the succinyl linker to release the material from the support and elimination of the terminal uridine as the 2',3'-cyclic phosphate. Although both steps, along with removal of base-protecting groups, can be performed simultaneously, elimination of the terminal cyclic phosphate is the rate-limiting step. After normal NH<sub>4</sub>OH deprotection, treatment at neutral pH with lead acetate (18 hr at 37°C) can complete the terminal deblocking (Gough et al., 1983). Complete deblocking can be performed with extended NH<sub>4</sub>OH hydrolysis, although the rate depends on the nature of the adjacent nucleoside with all ribonucleosides > dA, dG > T > dC (Debear et al., 1987). Therefore, NH<sub>4</sub>OH deprotection conditions ranging from 16 hr at 50°C to 24 hr at 65°C were first proposed, and a sub-

sequent paper has used 48 hr at 65°C (Schwartz et al., 1995). Attachment of a ribonucleotide through the  $N^4$ -position of a cytosine base (Fig. 3.1.7C), with subsequent chain extension from the 3'-phosphate group, has also been used (Pochet et al., 1987). In this case, cleavage was performed using 2N NaOH (10 min at 60°C); however, use of alkali hydroxides is not recommended because of possible damage to cytosine bases. A reversed uridine phosphoramidite reagent can also be used to convert previously derivatized supports into universal supports (Fig. 3.1.7D). Universal supports with N-benzoylcytidine linkers (Fig. 3.1.7Bd) are commercially available (Biogenex, San Ramon, CA), and the addition of 0.5 M lithium chloride to the NH<sub>4</sub>OH reagent has been recommended for their cleavage and deprotection (15 hr at 55°C).

A ribonucleoside is not essential for a universal linker, and other diol linkers have been used. An acyclic 3-amino-1,2-propanediol linker has been reported (Lyttle et al., 1996) that uses neighboring group participation by the amino group to cleave the oligonucleotide under mild conditions (0.1 M triethylamine acetate [1 mL] plus 3% NH<sub>4</sub>OH [40  $\mu$ L], 2 hr at room temperature; Fig. 3.1.7E). This linker,



**Figure 3.1.7** Universal linker arms for the synthesis of oligonucleotides with free 3'-OH ends. (**A**) First 3'-2'(3') inverted linkages (Crea and Horn, 1980; van der Marel et al., 1982). (**B**) Universal supports based on: (**a**) 2'(3')-O-benzoyluridine (Gough et al., 1983); (**b**) 2'(3')-O-acetyluridine (Cosstick and Eckstein, 1985); (**c**)  $N^3$ -anisoyluridine (Debear et al., 1987); (**d**) commercially available  $N^4$ -benzoylcytidine universal support (Biogenex, San Ramon, CA); (**e**) 1,4-anhydroribitol (Scheuerlarsen et al., 1997). (**C**) Inverted linkage attached through  $N^4$ -position of cytidine (Pochet et al., 1987). (**D**) Inverted linkage obtained through use of a "universal" 5'-phosphoramidite (Schwartz et al., 1995). (**E**) Acyclic universal linker with a 3-N-allyloxycarbonyl-protecting group (Lyttle et al., 1996). (**F**) Special phosphoramidite linkage to allow the synthesis of a second oligonucleotide on the 5'-end of another (Hardy et al., 1998). (**G**) 1,4-Anhydroerythreitol coupled via a succinyl linker to CPG (Nelson et al., 1997). DMT, 5'-dimethoxytrityl (DMTr).

however, required a preliminary step with tetrakis(triphenylphosphine) palladium to remove the *N*-allyloxycarbonyl-protecting group, and not all of the oligonucleotide was released from the support.

The rate of cleavage has been found to depend strongly on the stereochemistry of the attacking hydroxyl group, as reported in studies

that used the cyclic *cis* diol 1,4-anhydroerythritol (Scott et al., 1994; Hardy et al., 1998). These studies developed a novel linker, phosphoramidite, that allowed consecutive oligonucleotide sequences to be synthesized on the same support (Fig. 3.1.7F). Cleavage with NH<sub>4</sub>OH (16 hr at 80°C) or NH<sub>4</sub>OH/MeNH<sub>2</sub> (16 hr at 60°C) was recommended. The 1,4-anhy-

droerythritol linker has also been coupled to CPG supports through a succinic acid linker (Fig. 3.1.7G); these Rainbow Universal CPG supports are commercially available (Clontech, Palo Alto, CA). Deprotection and cleavage from the Rainbow Universal CPG supports was performed using either 0.5 M LiCl/NH<sub>4</sub>OH (16 hr at 55°C) or 0.23 M triethylamine/0.5 M LiCl/NH<sub>4</sub>OH (1 hr at 80°C) as the cleavage reagent (Nelson et al., 1997).

Deprotection of the hydroxyl group adjacent to the phosphate linkage is a requirement before this group can participate in the cyclization and phosphate group elimination reactions. Therefore, faster deprotection can be obtained when the very labile chloroacetyl-protecting group is used instead of acetyl, benzoyl, or succinyl groups (Scheuerlarsen et al., 1997). This strategy was first applied to a 1,4-anhydroribitol linker arm using a specific 2'-5' linkage (Fig. 3.1.7Be), and cleavage of the terminal cyclic phosphate was achieved under "normal" deprotection conditions (NH<sub>4</sub>OH for either 12 hr at 55°C or 72 hr at 22°C).

#### Linker Arms for the Deprotection of Immobilized Products with Optional Postdeprotection Cleavage

A number of applications require the removal of all or some of the protecting groups before the oligonucleotide is cleaved from the support (i.e., deprotection conditions orthogonal to cleavage conditions). Removal of the terminal 4'-dimethoxytrityl-protecting group is easily performed because the acidic conditions do not affect the acid-resistant linker arms most commonly used. In contrast, removal of the phosphate-protecting groups from the internucleotide linkages or the amino-protecting groups from the adenine, cytosine, and guanine bases requires special consideration if these groups are to be removed without cleaving the product from the solid-phase support. If, however, these deprotection steps are performed while the product remains immobilized (i.e., attached to the support), then the solid-phase support can provide the same handling and workup advantages as realized during the solidphase synthesis. After these steps, the linker arm can be cleaved to release the products. In certain cases, the final product will be used while immobilized on the support, but it is still helpful to be able to cleave samples off for characterization and quality analysis.

Generally, succinyl linkers are not sufficiently stable to allow removal of any protecting groups other than *O*-methyl- or cyanoethyl

phosphate-protecting groups. Succinyl linkers, however, can be left intact if different protecting group and deprotection schemes are employed. For example, hydrazine hydrate/pyridine/acetic acid can be used as the deprotection reagent if the N<sup>6</sup>-isobutyryl-protecting group on deoxyguanosine is replaced with an  $N^6$ -(N',N'-dibutylformamidine)-protecting group (Urdea and Horn, 1986). Base deprotection using ethanolamine can also be accomplished, but the  $N^4$ -protection on deoxycytidine must be modified to prevent  $N^4$ -hydroxyethylation (Berner et al., 1989). Allylic protection on all of the bases and on the phosphate linkages can also be removed using palladium reagents without affecting the succinyl linker (Hayakawa et al., 1990).

Attachment of a succinyl linker to a secondary N-methyl amino group on an intermediate linker such as N-methyl glycine (sarcosine), bis-1,6-dimethylaminohexane, or N-propyl polyethylene glycol (Fig. 3.1.8A to C) instead of a primary amino group (such as LCAA-CPG) creates linkages that are resistant to cleavage by the non-nucleophilic base DBU. These linkages have been used with base labile 5'-Fmoc-protecting groups to make acid-sensitive oligodeoxyribonucleotides and oligoribonucleotides (Brown et al., 1989) or with 2-(4-nitrophenyl)ethoxycarbonyl/NPE baseprotecting groups to allow on-column deprotection (Stengele and Pfleiderer, 1990; Weiler and Pfleiderer, 1995). A triethylamine-resistant sarcosine-succinic acid linker arm (Fig. 3.1.8D) has also been used to prepare branched RNA and DNA/RNA chimeras (Grotli et al., 1997). In each case, after synthesis or deprotection, the products are easily released from the support by conventional hydrolysis with NH₄OH.

A stronger linkage can also be created by attaching the succinyl linker to the amino group of a base instead of a hydroxyl group (Figs. 3.1.7C and 3.1.8E and F). Alkaline hydrolysis or oxidative cleavage with NaIO<sub>4</sub> can then release the product. This strategy, however, requires two orthogonal-protecting groups for the 5'- and 3'(2')-positions and does not offer any advantage, except for the synthesis of cyclic oligonucleotides.

A number of different linker arms have also been developed that take advantage of the resistance of phosphate ester and amide bonds to alkaline hydrolysis and allow protecting group removal before cleavage from the support. Thus thioether and thiophosphate linkers cleavable by oxidative cleavage (Fig. 3.1.8G and H), allyl

Figure 3.1.8 Linker arms that allow on-column deprotection and then optional cleavage. (A) Succinic acid linked to an *N*-methylglycine (sarcosine) derivatized support (Brown et al., 1989). (B) Succinic acid linked to 1,6-*bis* methylaminohexane spacer (Stengele and Pfleiderer, 1990). (C) Succinic acid linked to *N*-propyl polyethylene glycol Tentagel support (Weiler and Pfleiderer, 1995). (D) Succinyl-sarcosine linkage for the solid-phase synthesis of branched oligonucleotides (Grotli et al., 1997). (E) Linkage through the amino group of cytosine for branched and cyclic oligonucleotide synthesis (De Napoli et al., 1995). (F) Oxidizable solid support (Bower et al., 1987; Markiewicz et al., 1994). (G) Phenyl thioether linker, which is stable until oxidized into a phenylsulfone (Felder et al., 1984). (H) Thiophosphate linker, cleavable by iodine/water oxidation or acetic acid hydrolysis (Tanaka et al., 1989). (I) 3-Chloro-4-hydroxyphenyl linker for the solid-phase synthesis of cyclic oligonucleotides (Alazzouzi et al., 1997). (J) Linker arm produced from tolylene 2,6-diisocyanate with more stable carbamate and urethane linkages (Kumar, 1994; Sproat and Brown, 1985). DMT, 5'-dimethoxytrityl (DMTr).

linkers cleavable with tetrakis(triphenylphosphine) palladium (Fig. 3.1.5F), and phosphoroamidate linkages cleavable by acidic hydrolysis have been developed (Gryaznov and

Letsinger, 1992). These supports can also be considered as universal supports because the first nucleotide is added as part of the automated synthesis. All of these methods, how-

Figure 3.1.8 Continued

ever, produced oligonucleotides with a terminal 3'-phosphate instead of a 3'-hydroxyl group. An exception was the solid-phase synthesis of cyclic oligonucleotides. In this approach, 3chloro-4-hydroxylphenylacetic acid served as both the linker arm and a phosphate-protecting group (Fig. 3.1.8I). The phosphotriester linkage was converted into a phosphodiester by selective removal of the cyanoethyl-protecting group, and then cyclization of the 5'-end to the 3'-terminal phosphodiester group was achieved using 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole as the coupling agent. The linker was then cleaved with tetramethylguanidinium syn-2-aldoximate (8 hr), and a circular oligonucleotide was released (Alazzouzi et al., 1997). The requirement for a 3'-phosphate has also been avoided by insertion of a disiloxyl linkage between the nucleoside and the phosphate linkage (Fig. 3.1.4E). This linkage was stable to mild base, and preliminary purification by selective cleavage of apurinic sites using triethylamine/ethanol (1:1, 3 hr at 20°C), was possible (Kwiatkowski et al., 1996). After this treatment, the products were cleaved from the support with tetrabutylammonium fluoride and then deprotected with NH<sub>4</sub>OH.

Isocyanate reagents can react with hydroxyl groups and amino groups to produce carbamate and urethane bonds, respectively, which are more resistant to hydrolysis than are esters. Although acyclic diisocyanates have not been successful, the more rigid tolylene 2,6-diisocyanate reagent (Sproat and Brown, 1985) has been used to create a stable linker arm (Fig. 3.1.8J). The carbamate linkage can be hydrolyzed by long exposure to NH<sub>4</sub>OH (24 to 48 hr at 55°C), whereas deprotection under milder conditions allowed the product to remain attached to the support.

Two methods have been developed for removing failure or depurinated sequences from the full-length product while both are still attached to the support. In the first method (Urdea and Horn, 1986), spleen phosphodiesterase was used to selectively degrade non-full-length oli-

**Figure 3.1.9** Linker arms for permanent attachment to solid-phase supports. (**A**) Hydroxy propylamine linker (Seliger et al., 1995). (**B**) Dimethoxytrityl glycolic acid linker (Hakala et al., 1997). (**C**) Dimethoxytrityl-4,7,10,13-tetraoxatridecanoate linker (Markiewicz et al., 1994). (**D**) Long spacer linkages prepared using repetitive coupling of various phosphoramidites (Shchepinov et al., 1997). (**E**) Cleavable spacer linkage used in conjunction with the preceding phosphoramidites to control the surface oligonucleotide density (Shchepinov et al., 1997), (**F**) Direct phosphate linkage to surface silanol groups (Cohen et al., 1997). (**G**) Diol linker formed from 3-glycidoxypropyl trimethoxysilane (Maskos and Southern, 1992). (**H**) Polyethylene glycol linkers (Maskos and Southern, 1992). (**I**) *Bis*-(2-hydroxethyl)-aminopropylsilane linker with hexaethylene glycol spacer phosphoramidites (Pease et al., 1994). (**J**) *N*-(3-(triethoxysilyl)-propyl)-4-hydroxybutyramide linker (McGall et al., 1997). (**K**) Linkage through the *N*<sup>4</sup>-position of cytosine (Markiewicz et al., 1994). (**L**) Triethylene glycol ethylacrylamide linker (Markiewicz et al., 1994). DMT, 5'-dimethoxytrityl (DMTr).

gonucleotides. This method required a careful selection of protecting groups and a special capping reagent (levulinic anhydride) that could be selectively removed without exposing the full-length product (blocked by a DMTr or 5'-benzoyl group) to enzymatic digestion. In a second approach (Horn and Urdea, 1988; Kwiatkowski et al., 1996), the purification of 5'-dimethoxytritylated oligonucleotides was

improved by selective fragmentation of sequences containing apurinic defects, while still attached to the support. This treatment greatly reduced the proportion of DMTr-labeled oligonucleotides that were not full-length and allowed oligonucleotides as long as 118 bases to be purified by a simple reverse-phase cartridge procedure.

Figure 3.1.9 Continued

## **Linker Arms for Permanent Attachment to the Support**

Linker arms for permanent attachment of the oligonucleotide to the support must be very resistant to the hydrolysis conditions used to remove protecting groups. The most common method for permanent attachment is through phosphodiester or phosphoroamidate linkages by the direct coupling of phosphoramidite reagents, respectively, to hydroxyl or amino derivatized supports. The phosphoramidate linkages are particularly common when aminated polypropylene films are used as supports (Matson et al., 1994, 1995; Wehnert et al., 1994; Shchepinov et al., 1997). Other derivatized supports have been converted into hydroxyl functionalized materials using either 3-amino-1propanol, dimethoxytrityl glycolic acid, sodium

13-O-dimethoxytrityl-4,7,10,13-tetra-oxatrid ecanoate, or a variety of consecutive spacer

phosphoramidites (Fig. 3.1.9A to D). These spacer phosphoramidites may also contain cleavable sulfonyldiethanol groups (Fig. 3.1.9E) or positively charged 2-amino-1,2-propandiol groups so that the surface density and charge can be modulated as well as the spacer chain length.

Although a recent publication has described the direct coupling of phosphoramidite reagents to the silanol groups on acid-washed glass slides (Fig. 3.1.9F), most glass surfaces are derivatized with alkoxysilanes to produced linker arms extending away from the surface. Several hydroxy-derivatized linkers on glass based on 3-glycidoxypropyl trimethoxysilane and ethylene glycol ethers have been described (Fig. 3.1.9G and H). Unfortunately, however, these linkers were not completely resistant to NH<sub>4</sub>OH (5 to 10 hr at 55°C), and much of the product was lost. Other hydroxyl linker arms on glass plates have been based on *bis*-(2-hy-

droxyethyl)-aminopropylsilane or N-(3-(triethoxysilyl)-aminopropyl)-4-hydroxybutyramide and used in the synthesis of high-density oligonucleotide arrays (Fig. 3.1.9I and J). In these reports, base deprotection was accomplished using 1,2-diaminoethane/ethanol (1:1, 2 to 6 hr at room temperature) instead of hot NH<sub>4</sub>OH, and no linker cleavage was reported. Linkages to CPG beads using attachments through the  $N^4$ -position of 2'-deoxycytidine or through a triethylene glycol ethylacrylamide linker have also been reported (Fig. 3.1.9K and L); however, the researchers noted that the lability of the disiloxane bond (Si-O-Si) between any silica-based support and silane linker arm is a limiting factor and, although certain resistance to aqueous NH<sub>4</sub>OH is possible, use of pyridine/NH<sub>4</sub>OH always results in substantial cleavage (Markiewicz et al., 1994).

#### CONCLUSIONS

Solid-phase oligonucleotide synthesis was once considered to be a "mature" technology limited to incremental improvements; however, new and ingenious applications for oligonucleotide based materials continue to be developed, and the role of solid-phase synthesis is clearly going to be very important in making these new materials available. Quite remarkably, the scale of these applications spans the range from the extremely minute (i.e., single molecule detection and nanoengineering) to large-scale oligonucleotide pharmaceuticals. Success in any of these areas requires a firm understanding of the chemical requirements of every step involved. It is hoped that this short review provides enough introduction to convey the power and diversity of solid-phase oligonucleotide synthesis techniques and convince the reader that new technology can be developed in even mature fields.

#### **Literature Cited**

- Adinolfi, M., Barone, G., Denapoli, L., Iadonisi, A., and Piccialli, G. 1996. Solid phase synthesis of oligosaccharides. *Tetrahedron Lett.* 37:5007-5010.
- Alazzouzi, E., Escaja, N., Grandas, A., and Pedroso, E. 1997. A straightforward solid-phase synthesis of cyclic oligodeoxyribonucleotides. *Angew. Chem. Intl. Ed. Engl.* 36:1506-1508.
- Albretsen, C., Kalland, K.-H., Haukanes, B.-I., Håvarstein, L.-S., and Kleppe, K. 1990. Applications of magnetic beads with covalently attached oligonucleotides in hybridization: Isolation and detection of specific measles virus mRNA from a crude cell lysate. *Anal. Biochem.* 189:40-50.

- Alul, R.H., Singman, C.N., Zhang, G.R., and Letsinger, R.L. 1991. Oxalyl-CPG—A labile support for synthesis of sensitive oligonucleotide derivatives. *Nucl. Acids Res.* 19:1527-1532.
- Arnold, L., Tocík, Z., Bradková, E., Hostomský, Z., Paces, V., and Smrt, J. 1989. Automated chloridite and amidite synthesis of oligodeoxyribonucleotides on a long chain support using amidine protected purine nucleosides. *Collect. Czech. Chem. Commun.* 54:523-532.
- Asseline, U. and Thuong, N.T. 1989. Solid-phase synthesis of modified oligodeoxyribonucleotides with an acridine derivative or a thiophosphate group at their 3' end. *Tetrahedron Lett.* 30:2521-2524.
- Avino, A., Garcia, R.G., Diaz, A., Albericio, F., and Eritja, R. 1996. A comparative study of supports for the synthesis of oligonucleotides without using ammonia. *Nucleos. Nucleot.* 15:1871-1889.
- Bader, R., Brugger, H., Hinz, M., Rembe, C., Hofer, E.P., and Seliger, H. 1997a. A rapid method for the preparation of a one dimensional sequenceoverlapping oligonucleotide library. *Nucleos. Nucleot.* 16:835-842.
- Bader, R., Hinz, M., Schu, B., and Seliger, H. 1997b. Oligonucleotide microsynthesis of a 200-mer and of one dimensional arrays on a surface of hydroxylated polypropylene tape. *Nucleos. Nucleot.* 16:829-833.
- Bardella, F., Eritja, R., Pedroso, E., and Giralt, E. 1993. Gel-phase P-31 NMR—A new analytical tool to evaluate solid phase oligonucleotide synthesis. *Bioorgan. Med. Chem. Lett.* 3:2793-2796.
- Beattie, K.L., Logsdon, N.J., Anderson, R.S., Espinosa-Lara, J.M., Maldonado-Rodriguez, R., and Frost, J.D.I. 1988. Gene synthesis technology: Recent developments and future prospects. *Biotechnol. Appl. Biochem.* 10:510-521.
- Beaucage, S.L. and Iyer, R.P. 1992. Advances in the synthesis of oligonucleotides by the phosphoramidite approach. *Tetrahedron* 48:2223-2311.
- Beaucage, S.L. and Iyer, R.P. 1993. The functionalization of oligonucleotides via phosphoramidite derivatives. *Tetrahedron* 49:1925-1963.
- Belagaje, R. and Brush, C.K. 1982. Polymer supported synthesis of oligonucleotides by a phosphotriester method. *Nucl. Acids Res.* 10:6295-6303.
- Bergmann, F. and Bannwarth, W. 1995. Solid phase synthesis of directly linked peptide-oligode-oxynucleotide hybrids using standard synthesis protocols. *Tetrahedron Lett.* 36:1839-1842.
- Berner, S., Gröger, G., and Seliger, H. 1989. A new option in solid phase synthesis of DNA fragments. *Nucleos. Nucleot.* 8:1165-1167.
- Bhongle, N.N. and Tang, J.Y. 1995. A convenient and practical method for derivatization of solid supports for nucleic acid synthesis. *Synth. Commun.* 25:3671-3679.

- Birch-Hirschfield, E., Foldespapp, Z., Guhrs, K.H., and Seliger, H. 1996. Oligonucleotide synthesis on polystyrene-grafted poly(tetrafluoroethylene) support. Helv. Chim. Acta 79:137-150.
- Boal, J.H., Wilk, A., Harindranath, N., Max, E.E., Kempe, T., and Beaucage, S.L. 1996. Cleavage of oligodeoxyribonucleotides from controlledpore glass supports and their rapid deprotection by gaseous amines. Nucl. Acids Res. 24:3115-3117.
- Bonora, G.M. 1995. Polyethylene glycol. A high efficiency liquid phase (HELP) for the large scale synthesis of the oligonucleotides. Appl. Biochem. Biotechnol. 54:3-17.
- Bonora, G.M., Baldan, A., Schiavon, O., Ferruti, P., and Veronese, F.M. 1996. Poly(N-acryloylmorpholine) as a new soluble support for the liquidphase synthesis of oligonucleotides. Tetrahedron Lett. 37:4761-4764.
- Bower, M., Summers, M.F., Kell, B., Hoskins, J., Zon, G., and Wilson, W.D. 1987. Synthesis and characterization of oligodeoxyribonucleotides containing terminal phosphates. NMR, UV spectroscopic and thermodynamic analysis of duplex formation of [d(pGGATTCC)]2, [d(GGAAT-TCCp)<sub>2</sub> and [d(pGGAATTCCp)]<sub>2</sub>. Nucl. Acids Res. 15:3531-3547.
- Brown, T., Pritchard, C.E., Turner, G., and Salisbury, S.A. 1989. A new base-stable linker for solidphase oligonucleotide synthesis. J. Chem. Soc. Chem. Commun. 891-893.
- Caruthers, M.H. 1991. Chemical synthesis of DNA and DNA analogues. Acc. Chem. Res. 24:278-
- Chow, F. and Kempe, T. 1997. Process and reagents for processing synthetic oligonucleotides. United States Patent #5,656,741.
- Chu, T.J., Caldwell, K.D., Weiss, R.B., Gesteland, R.F., and Pitt, W.G. 1992. Low fluorescence background electroblotting membrane for DNA sequencing. Electrophoresis 13:105-114.
- Cohen, G., Deutsch, J., Fineberg, J., and Levine, A. 1997. Covalent attachment of DNA oligonucleotides to glass. Nucl. Acids Res. 25:911-912.
- Cosstick, R. and Eckstein, F. 1985. Synthesis of d(GC) and d(CG) octamers containing alternating phosphorothioate linkages: Effect of the phosphorothioate group on the B-Z transition. Biochemistry 24:3630-3638.
- Cramer, F., Helbig, R., Hettler, H., Scheit, K.H., and Seliger, H. 1966. Oligonucleotide synthesis with a soluble polymer as a carrier. Angew. Chem. Intl. Ed. Engl. 5:601-601.
- Crea, R. and Horn, T. 1980. Synthesis of oligonucleotides on cellulose by a phosphotriester method. Nucl. Acids Res. 8:2331-2348.
- Damha, M.J., Giannaris, P.A., and Zabarylo, S.V. 1990. An improved procedure for derivatization of controlled pore glass beads for solid-phase oligonucleotide synthesis. Nucl. Acids Res. 18:3813-3821.

- Debear, J.S., Hayes, J.A., Koleck, M.P., and Gough, G.R. 1987. A universal glass support for oligonucleotide synthesis. Nucleos. Nucleot. 6:821-830.
- Dell'Aquila, C., Imbach, J.L., and Rayner, B. 1997. Photolabile linker for the solid-phase synthesis of base-sensitive oligonucleotides. Tetrahedron Lett. 38:5289-5292.
- De Napoli, L., Galeone, A., Mayol, L., Messere, A., Montesarchio, D., and Piccialli, G. 1995. Automated solid phase synthesis of cyclic oligonucleotides: A further improvement. Bioorgan. Med. Chem. 3:1325-1329.
- Devivar, R.V., Koontz, S.L., Peltier, W.J., Pearson, J.E., Guillory, T.A., and Fabricant, J.D. 1999. A new solid-support for oligonucleotide synthesis. Biorg. Med. Chem. Lett. 9:1239-1242.
- Duncan, C.H. and Cavalier, S.L. 1988. Affinity chromatography of a sequence-specific DNA binding protein using Teflon-linked oligonucleotides. Anal. Biochem. 169:104-108.
- Efcavitch, J.W., McBride, L.J., and Eadie, J.S. 1986. Effect of pore diameter on the support-bound synthesis of long oligodeoxynucleotides. In Biophosphates and Their Analogues-Synthesis, Structure, Metabolism and Activity (K.S. Bruzik and W.J. Stec, eds.) pp. 65-70. Elsevier Science Publishing, New York.
- Efimov, V.A., Buryakova, A.A., Reverdatto, S.V., Chakhmakhcheva, O.G., and Ovchinnikov, Y.A. 1983. Rapid synthesis of long-chain deoxyribooligonucleotides by the N-methylimidazole phosphotriester method. Nucl. Acids Res. 11:8369-8387.
- Eritja, R., Robles, J., Fernandezforner, D., Albericio, F., Giralt, E., and Pedroso, E. 1991. NPE-resin, a new approach to the solid-phase synthesis of protected peptides and oligonucleotides. 1. Synthesis of the supports and their application to oligonucleotide synthesis. Tetrahedron Lett. 32:1511-1514.
- Felder, E., Schwyzer, R., Charubala, R., Pfleiderer, W., and Schulz, B. 1984. A new solid phase approach for rapid synthesis of oligonucleotides bearing a 3'-terminal phosphate group. Tetrahedron Lett. 25:3967-3970.
- Fodor, S.P.A., Read, J.L., Pirrung, M.C., Stryer, L., Lu, T.L., and Solas, D. 1991. Light-directed, spatially addressable parallel chemical synthesis. Science 251:767-773.
- Frank, R. 1993. Strategies and techniques in simultaneous solid phase synthesis based on the segmentation of membrane type supports. Bioorgan. Med. Chem. Lett. 3:425-430.
- Frank, R., Heikens, W., Heisterberg-Moutsis, G., and Blocker, H. 1983. A new general approach for the simultaneous chemical synthesis of large numbers of oligonucleotides: Segmental solid supports. Nucl. Acids Res. 13:4365-4377.
- Fruchtel, J.S. and Jung, G. 1996. Organic chemistry on solid supports. Angew. Chem. Intl. Ed. Engl. 35:17-42.

- Gait, M.J., Matthes, H.W.D., Singh, M.S., Sproat, B.S., and Titmas, R.C. 1982. Rapid synthesis of oligodeoxyribonucleotides VII. Solid phase synthesis of oligodeoxyribonucleotides by a continuous flow phosphotriester method on a kieselguhr-polyamide support. *Nucl. Acids Res.* 10:6243-6254.
- Gold, L., Polisky, B., Uhlenbeck, O., and Yarus, M. 1995. Diversity of oligonucleotide functions. Annu. Rev. Biochem. 64:763-797.
- Gough, G.R., Brunden, M.J., and Gilham, P.T. 1983. 2'(3')-O-Benzoyluridine 5' linked to glass: An all purpose support for solid phase synthesis of oligodeoxyribonucleotides. *Tetrahedron Lett.* 24:5321-5324.
- Gray, D.E., Case-Green, S.C., Fell, T.S., Dobsen, P.J., and Southern, E.M. 1997. Ellipsometric and interferometric characterization of DNA probes immobilized on a combinatorial array. *Langmuir* 13:2833-2842.
- Greenberg, M.M. and Gilmore, J.L. 1994. Cleavage of oligonucleotides from solid-phase supports using *O*-nitrobenzyl photochemistry. *J. Org. Chem.* 59:746-753.
- Grotli, M., Eritja, R., and Sproat, B. 1997. Solid phase synthesis of branched RNA and branched DNA/RNA chimeras. *Tetrahedron* 53:11317-11347.
- Gryaznov, S.M. and Letsinger, R.L. 1992. A new approach to synthesis of oligonucleotides with 3' phosphoryl groups. *Tetrahedron Lett.* 33:4127-4128.
- Gupta, K.C., Sharma, P., Kumar, P., and Sathyanarayana, S. 1991. A general method for the synthesis of 3'-sulfhydryl and phosphate group containing oligonucleotides. *Nucl. Acids Res.* 19:3019-3025.
- Guzaev, A. and Lonnberg, H. 1997. A novel solid support for synthesis of 3'-phosphorylated chimeric oligonucleotides containing internucleosidic methyl phosphotriester and methylphosphonate linkages. *Tetrahedron Lett.* 38:3989-3992.
- Hakala, H., Heinonen, P., Iitia, A., and Lonnberg, H. 1997. Detection of oligonucleotide hybridization on a single microparticle by time-resolved fluorometry: Hybridization assays on polymer particles obtained by direct solid phase assembly of the oligonucleotide probes. *Bioconjugate Chem.* 8:378-384.
- Hardy, P.M., Holland, D., Scott, S., Garman, A.J., Newton, C.R., and McLean, M.J. 1998. Reagents for the preparation of two oligonucleotides per synthesis (TOPS). *Nucl. Acids Res.* 22:2998-3004.
- Hayakawa, Y., Wakabayashi, S., Kato, H., and Noyori, R. 1990. The allylic protection method in solid-phase oligonucleotide synthesis: An efficient preparation of solid-anchored DNA oligomers. J. Am. Chem. Soc. 112:1691-1696.
- Hayatsu, H. and Khorana, H.G. 1966. Deoxyribooligonucleotide synthesis on a polymer support. *J. Am. Chem. Soc.* 88:3182-3183.

- Hermes, J.D., Parekh, S.M., Blacklow, S.C., Köster, H., and Knowles, J.R. 1989. A reliable method for random mutagenesis: The generation of mutant libraries using spiked oligodeoxyribonucleotide primers. *Gene* 84:143-151.
- Horn, T. and Urdea, M.S. 1988. Solid-supported hydrolysis of apurinic sites in synthetic oligonucleotides for rapid and efficient purification on reverse- phase cartridges. *Nucl. Acids Res.* 16:11559-11571.
- Hyrup, B. and Nielsen, P.E. 1996. Peptide nucleic acids (PNA): synthesis, properties and potential applications. *Bioorgan. Med. Chem.* 4:5-23.
- Ito, H., Ike, Y., Ikuta, S., and Itakura, K. 1982. Solid phase synthesis of polynucleotides. VI. Further studies on polystyrene copolymers for the solid support. *Nucl. Acids Res.* 10:1755-1769.
- Kamaike, K., Hasegawa, Y., and Ishido, Y. 1988. Efficient synthesis of an oligonucleotide on a cellulose acetate derivative as a novel polymersupport using phosphotriester approach. *Tetra-hedron Lett.* 29:647-650.
- Katzhendler, J., Cohen, S., Weisz, M., Ringel, I., Camerini-Oterio, R.D., and Deutsch, J. 1987. Spacer effect on the synthesis of oligonucleotides by the phosphite method. *Reactive Polymers* 6:175-187.
- Katzhendler, J., Cohen, S., Rahamim, E., Weisz, M., Ringel, I., and Deutsch, J. 1989. The effect of spacer, linkage and solid support on the synthesis of oligonucleotides. *Tetrahedron* 45:2777-2792.
- Khorana, H.G. 1979. Total synthesis of a gene. *Science* 203:614-625.
- Kumar, A. 1994. Development of a suitable linkage for oligonucleotide synthesis and preliminary hybridization studies on oligonucleotides synthesized in situ. *Nucleos. Nucleot.* 13:2125-2134.
- Kumar, P., Bose, N.K., and Gupta, K.C. 1991. A versatile solid phase method for the synthesis of oligonucleotide-3'-phosphates. *Tetrahedron Lett.* 32:967-970.
- Kumar, P., Sharma, A.K., Sharma, P., Garg, B.S., and Gupta, K.C. 1996. Express protocol for functionalization of polymer supports for oligonucleotide synthesis. *Nucleos. Nucleot.* 15:879-
- Kwiatkowski, M., Nilsson, M., and Landegren, U. 1996. Synthesis of full-length oligonucleotides: Cleavage of apurinic molecules on a novel support. *Nucl. Acids Res.* 24:4632-4638.
- Lipshutz, R.J., Morris, D., Chee, M., Hubbell, E., Kozal, M.J., Shah, N., Shen, N., Yang, R., and Fodor, S.P.A. 1995. Using oligonucleotide probe arrays to access genetic diversity. *BioTechniques* 19:442-447.
- Lyttle, M.H., Hudson, D., and Cook, R.M. 1996. A new universal linker for solid phase DNA synthesis. *Nucl. Acids Res.* 24:2793-2798.

- 7128
- Macdonald, P.M., Damha, M.J., Ganeshan, K., Braich, R., and Zabarylo, S.V. 1996. Phosphorus 31 solid state NMR characterization of oligonucleotides covalently bound to a solid support. *Nucl. Acids Res.* 24:2868-2876.
- Macmillan, A.M. and Verdine, G.L. 1991. Engineering tethered DNA molecules by the convertible nucleoside approach. *Tetrahedron* 47:2603-2616.
- Markiewicz, W.T. and Wyrzykiewicz, T.K. 1989. Universal solid supports for the synthesis of oligonucleotides with terminal 3'-phosphates. *Nucl. Acids Res.* 17:7149-7158.
- Markiewicz, W.T., Adrych-Rozek, K., Markiewicz, M., Zebrowska, A., and Astriab, A. 1994. Synthesis of oligonucleotides permanently linked with solid supports for use as synthetic oligonucleotide combinatorial libraries. *In* Innovation and Perspectives in Solid Phase Synthesis: Peptides, Proteins and Nucleic Acids: Biological and Biomedical Applications (R. Epton, ed.) pp. 339-346. Mayflower Worldwide, Birmingham.
- Maskos, U. and Southern, E.M. 1992. Oligonucleotide hybridizations on glass supports: A novel linker for oligonucleotide synthesis and hybridisation properties of oligonucleotides synthesized in situ. *Nucl. Acids Res.* 20:1679-1684.
- Matson, R.S., Rampal, J.B., and Coassin, P.J. 1994. Biopolymer synthesis on polypropylene supports. *Anal. Biochem.* 217:306-310.
- Matson, R.S., Rampal, J., Pentoney, S.L., Jr., Anderson, P.D., and Coassin, P. 1995. Biopolymer synthesis on polypropylene supports: Oligonucleotide arrays. *Anal. Biochem.* 224:110-116.
- Matthes, H.W.D., Zenke, W.M., Grundström, T., Staub, A., Wintzerith, M., and Chambon, P. 1984. Simultaneous rapid chemical synthesis of over one hundred oligonucleotides on a microscale. *EMBO J.* 3:801-805.
- McCollum, C. and Andrus, A. 1991. An optimized polystyrene support for rapid, efficient oligonucleotide synthesis. *Tetrahedron Lett.* 32:4069-4072.
- McGall, G., Labadie, J., Brock, P., Wallraff, G., Nguyen, T., and Hinsberg, W. 1996. Light-directed synthesis of high-density oligonucleotide arrays using semiconductor photoresists. *Proc. Natl. Acad. Sci. U.S.A.* 93:13555-13560.
- McGall, G.H., Barone, A.D., Diggelmann, M., Fodor, S.P.A., Gentalen, E., and Ngo, N. 1997. The efficiency of light-directed synthesis of DNA arrays on glass substrates. *J. Am. Chem. Soc.* 119:5081-5090.
- Merrifield, R.B. 1965. Automated synthesis of peptides. *Science* 150:178-185.
- Milner, N., Mir, K.U., and Southern, E.M. 1997. Selecting effective antisense reagents on combinatorial oligonucleotide arrays. *Nature Biotechnol*. 15:537-541.
- Montserrat, F.X., Grandas, A., and Pedroso, E. 1993.
  Predictable and reproducible yields in the anchoring of DMT-nucleoside-succinates to highly loaded aminoalkyl-polystyrene. *Nucleos. Nucleot.* 12:967-971.

- Montserrat, F.X., Grandas, A., Eritja, R., and Pedroso, E. 1994. Criteria for the economic large scale solid-phase synthesis of oligonucleotides. *Tetrahedron* 50:2617-2622.
- Mullah, B., Livak, K., Andrus, A., and Kenney, P. 1998. Efficient synthesis of double dye-labeled oligodeoxyribonucleotide probes and their application in a real time PCR assay. *Nucl. Acids Res.* 26:1026-1031.
- Nelson, P.S., Muthini, S., Vierra, M., Acosta, L., and Smith, T.H. 1997. Rainbow™ universal CPG: A versatile solid support for oligonucleotide synthesis. *BioTechniques* 22:752-756.
- Ohsima, S.-I., Morita, K., and Takaku, H. 1984. Solid-phase synthesis of deoxyribooligonucleotides by the phosphotriester method employing a new polymer support. *Chem. Pharm. Bull.* 32:4690-4693.
- Ott, J. and Eckstein, F. 1984. Filter disc supported oligonucleotide synthesis by the phosphite method. *Nucl. Acids Res.* 12:9137-9142.
- Pease, A.C., Solas, D., Sullivan, E.J., Cronin, M.T., Holmes, C.P., and Fodor, S.P.A. 1994. Lightgenerated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Natl. Acad. Sci. U.S.A.* 91:5022-5026.
- Pochet, S., Huyn-Dinh, T., and Igolen, J. 1987. Synthesis of DNA fragments linked to a solid phase support. *Tetrahedron* 43:3481-3490.
- Pon, R.T. 1993. Solid-phase supports for oligonucleotide synthesis. *In* Protocols for Oligonucleotides and Analogs (S. Agrawal, ed.) pp. 465-496. Humana Press, Totowa, N.J.
- Pon, R.T. and Yu, S. 1997a. Hydroquinone-*O*,*O*′-diacetic acid ('Q-linker') as a replacement for succinyl and oxalyl linker arms in solid phase oligonucleotide synthesis. *Nucl. Acids Res.* 25:3629-3635.
- Pon, R.T. and Yu, S. 1997b. Rapid automated derivatization of solid-phase supports for oligonucleotide synthesis using uronium or phosphonium coupling reagents. *Tetrahedron Lett.* 38:3331-3334.
- Pon, R.T., Usman, N., and Ogilvie, K.K. 1988. Derivatization of controlled pore glass beads for solid phase oligonucleotide synthesis. *BioTechniques* 6:768-775.
- Pon, R.T., Buck, G.A., Hager, K.M., Naeve, C.W., Niece, R.L., Robertson, M., and Smith, A.J. 1996. Multi-facility survey of oligonucleotide synthesis and an examination of the performance of unpurified primers in automated DNA sequencing. *BioTechniques* 21:680-685.
- Pon, R.T., Yu, S., Guo, Z., Yang, X., and Sanghvi, Y.S. 1998. Reusable solid-phase supports for oligonucleotide synthesis using hydroquinone-O,O'-diacetic acid (Q-linker). Nucleos. Nucleot. In press.
- Pon, R.T., Yu, S., Guo, Z., and Sanghvi, Y.S. 1999. Multiple oligodeoxyribonucleotide syntheses on a reusable solid-phase CPG support via the hydroquinone-*O*, *O'*-diacetic acid (Q-linker) linker arm. *Nucl. Acids Res.* 27:1531-1538.

- Porco, J.A., Deegan, T., Devonport, W., Gooding, O.W., Heisler, K., Labadie, J.W., Newcomb, B., Nguyen, C., van Eikeren, P., Wong, J., and Wright, P. 1997. Automated chemical synthesis: From resins to instruments. *Mol. Divers.* 2:197-206
- Potapov, V.K., Veiko, V.P., Korolev, O.N., and Shabarova, Z.A. 1979. Rapid synthesis of oligodeoxyribonucleotides on a grafted polymer support. *Nucl. Acids Res.* 6:2041-2057.
- Rapp, W. 1996. PEG grafted polystyrene tentacle polymers: Physico-chemical properties and application in chemical synthesis. *In* Combinatorial Peptide and Non-Peptide Libraries: A Handbook (G. Jung, ed.) pp. 425-464. VCH, Weinbeim
- Rapp, W.E. 1997. Macro beads as microreactors: New solid-phase synthesis methodology. *In* Combinatorial Chemistry: Synthesis and Application, (S.R. Wilson and A.W. Czarnik, eds.) pp. 65-93. John Wiley & Sons, New York.
- Reddy, M.P., Hanna, N.B., and Farooqui, F. 1994a. Fast cleavage and deprotection of oligonucleotides. *Tetrahedron Lett.* 35:4311-4314.
- Reddy, M.P., Michael, M.A., Farooqui, F., and Girgis, S. 1994b. New and efficient solid support for the synthesis of nucleic acids. *Tetrahedron Lett.* 35:5771-5774.
- Routledge, A., Wallis, M.P., Ross, K.C., and Fraser, W. 1995. A new deprotection strategy for automated oligonucleotide synthesis using a novel silyl-linked solid support. *Bioorgan. Med. Chem. Lett.* 5:2059-2064.
- Scheuerlarsen, C., Rosenbohm, C., Jorgensen, T.J.D., and Wengel, J. 1997. Introduction of a universal solid support for oligonucleotide synthesis. *Nucleos. Nucleot.* 16:67-80.
- Schwartz, M.E., Breaker, R.R., Asteriadis, G.T., and Gough, G.R. 1995. A universal adapter for chemical synthesis of DNA or RNA on any single type of solid support. *Tetrahedron Lett.* 36:27-30.
- Schwyzer, R., Felder, E., and Failli, P. 1984. 148. The CAMET and CASET links for the synthesis of protected oligopeptides and oligodeoxynucleotides on solid and soluble supports. *Helv. Chim. Acta* 67:1316-1327.
- Scott, S., Hardy, P., Sheppard, R.C., and McLean, M.J. 1994. A universal support for oligonucleotide synthesis. *In* Innovation and Perspectives in Solid-Phase Synthesis. Peptides, Proteins, and Nucleic Acids, Biological and Biomedical Applications (R. Epton, ed.) pp. 115-124. Mayflower Worldwide, Ltd., Birmingham, IJK
- Seliger, H., Herold, A., Kotschi, U., Lyons, J., and Schmidt, G. 1987. Semi-mechanized simultaneous synthesis of multiple oligonucleotide fragments. *In Biophosphates and Their Analogues*— Synthesis, Structure, Metabolism and Activity (K.S. Bruzik and W.J. Stec, eds.) pp. 43-58. Elsevier Science Publishers, New York.

- Seliger, H., Kotschi, U., Scharpf, C., Martin, R., Eisenbeiss, F., Kinkel, J.N., and Unger, K.K. 1989. Polymer support synthesis XV. Behaviour of non-porous surface coated silica gel microbeads in oligonucleotide synthesis. *J. Chro*matogr. 476:49-57.
- Seliger, H., Bader, R., Birch-Hirschfield, E., Földes-Papp, Z., Hinz, M., and Scharpf, C. 1995. Surface reactive polymers for special applications in nucleic acid synthesis. *Reactive Functional Polymers* 26:119-126.
- Seliger, H., Bader, R., Hinz, M., Rotte, B., Astriab, A., Markiewicz, M., and Markiewicz, W.T. 1997. Synthetic oligonucleotide combinatorial libraries—Tools for studying nucleic acid interactions. *Nucleos. Nucleot.* 16:703-710.
- Sharma, P., Sharma, A.K., Malhotra, V.P., and Gupta, K.C. 1992. One pot general method for the derivatization of polymer support for oligonucleotide synthesis. *Nucl. Acids Res.* 20:4100-4100.
- Shchepinov, M.S., CaseGreen, S.C., and Southern, E.M. 1997. Steric factors influencing hybridisation of nucleic acids to oligonucleotide arrays. *Nucl. Acids Res.* 25:1155-1161.
- Sproat, B.S. and Brown, D.M. 1985. A new linkage for solid phase synthesis of oligodeoxyribonucleotides. *Nucl. Acids Res.* 13:2979-2987.
- Stengele, K.P. and Pfleiderer, W. 1990. Improved synthesis of oligodeoxyribonucleotides. *Tetra-hedron Lett.* 31:2549-2552.
- Stimpson, D.I., Hoijer, J.V., Hsieh, W.T., Jou, C., Gordon, J., Theriault, T., Gamble, R., and Baldeschwieler, J.D. 1995. Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides. *Proc. Natl. Acad. Sci. U.S.A.* 92:6379-6383.
- Tanaka, T., Yamada, Y., Uesugi, S., and Ikehara, M. 1989. Preparation of a new phosphorylating agent: *S*-(*N*-monomethoxytritylaminoethyl)-*O*-(*o*-chlorophenyl)phosphorothioate and its application in oligonucleotide synthesis. *Tetrahedron* 45:651-660.
- Tang, J.Y. and Tang, J.X. 1997. Passivated polymer supports for nucleic acid synthesis. United States Patent #5,668,268.
- Uddin, A.H., Piunno, P.A., Hudson, R.H., Damha, M.J., and Krull, U.J. 1997. A fiber optic biosensor for fluorimetric detection of triple-helical DNA. *Nucl. Acids Res.* 25:4139-4146.
- Urdea, M.S. and Horn, T. 1986. Solid-supported synthesis, deprotection and enzymatic purification of oligodeoxyribonucleotides. *Tetrahedron Lett.* 27:2933-2936.
- van Aerschot A., Herdewijn, P., and Vanderhaeghe, H. 1988. Silica gel functionalised with different spacers as solid support for oligonucleotide synthesis. *Nucleos. Nucleot.* 7:75-90.
- van der Laan, A.C., Brill, R., Kuimelis, R.G., Kuylyeheskiely, E., Vanboom, J.H., Andrus, A., and Vinayak, R. 1997. A convenient automated solid-phase synthesis of PNA-(5')-DNA-(3')-PNA chimera. *Tetrahedron Lett.* 38:2249-2252.

7130

- van der Marel, G.A., Marugg, J.E., de Vroom, E., Wille, G., Tromp, M., van Boeckel, C.A.A., and van Boom, J.H. 1982. Phosphotriester synthesis of DNA fragments on cellulose and polystyrene solid supports. *Recl. Trav. Chim. Pays-Bas* 101:234-241.
- Venkatesan, H. and Greenberg, M.M. 1996. Improved utility of photolabile solid phase synthesis supports for the synthesis of oligonucleotides containing 3'-hydroxyl termini. J. Org. Chem. 61:525-529.
- Wehnert, M.S., Matson, R.S., Rampal, J.B., Coassin, P., and Caskey, C.T. 1994. A rapid scanning strip for tri- and dinucleotide short tandem repeats. *Nucl. Acids Res.* 22:1701-1704.
- Weiler, J. and Hoheisel, J.D. 1996. Combining the preparation of oligonucleotide arrays and synthesis of high-quality primers. *Anal. Biochem.* 243:218-227.
- Weiler, J. and Hoheisel, J.D. 1997. Picomole syntheses of high quality oligonucleotide primers in combination with the preparation of oligonucleotide arrays. *Nucleos. Nucleot.* 16:1793-1796

- Weiler, J. and Pfleiderer, W. 1995. An improved method for the large scale synthesis of oligonucleotides applying the NPE/NPEOC strategy. *Nucleos. Nucleot.* 14:917-920.
- Winter, M. 1996. Supports for solid-phase organic synthesis. *In* Combinatorial Peptide and Non-Peptide Libraries: A Handbook (G. Jung, ed.) pp. 465-510. VCH, Weinheim.
- Wright, P., Lloyd, D., Rapp, W., and Andrus, A. 1993. Large scale synthesis of oligonucleotides via phosphoramidite nucleosides and a high-loaded polystyrene support. *Tetrahedron Lett.* 34:3373-3376.
- Yip, K.F. and Tsou, K.C. 1971. A new polymer support method for the synthesis of ribooligonucleotide. *J. Am. Chem. Soc.* 93:3272-3276.
- Zhang, X.H. and Jones, R.A. 1996. A universal allyl linker for solid-phase synthesis. *Tetrahedron Lett.* 37:3789-3790.

Contributed by Richard T. Pon University of Calgary Calgary, Alberta, Canada

# EXHIBIT 8

Volume 7, Number 3, June 1997 ISSN 1087-2906

# Antisense & Nucleic Acid Drug Development

(The Antisense Journal)

Editors:

Arthur M. Krieg, M.D. C. A. Stein, M.D., Ph.D.

UN PHARMAGY LIBBARY

89052685468

Mary Ann Liebert, Inc. publishers

NOW BIMONTHI

## Case 1:21-cv-01015-JLH Document 174-2 Filed 03/20/23 Page 32 of 318 PageID #: GENERAL INFORMATION

Antisense & Nucleic Acid Drug Development, a bimonthly journal, discusses human-made substances and their effects on gene expression at the RNA and DNA levels. It provides a forum for basic researchers in molecular and cell biology, chemical synthesis, and applied therapeutics to discuss the development of new concepts and experimental approaches to understand and modulate gene activity.

Antisense & Nucleic Acid Drug Development (ISSN: 1087–2906) is owned and published bimonthly by Mary Ann Liebert, Inc., 2 Madison Avenue, Larchmont, NY 10538. Telephone: (914) 834-3100; fax: (914) 834-3582; e-mail: liebert@pipeline.com Copyright © 1997 by Mary Ann Liebert, Inc. Printed in the United States of America.

**Postmaster:** Send address changes to *Antisense & Nucleic Acid Drug Development* c/o Subscription Department, Mary Ann Liebert, Inc., 2 Madison Avenue, Larchmont, NY 10538.

Subscriptions should be addressed to the Publisher and are payable in advance. Rates for subscriptions are \$148 per volume of 6 issues in the United States and Possessions, and \$196 elsewhere. Subscriptions begin with the first issue of the current volume. Bulk subscriptions available upon request from the Publisher.

Reprints, except special orders of 100 or more, are available from the authors.

Information for Manuscript Submission is given elsewhere in the publication.

Business Communications should be addressed to the Publisher.

Advertising inquiries from within the United States or Canada should be addressed to Mary Ann Liebert, Inc., 2 Madison Avenue, Larchmont, NY 10538; telephone (914) 834-3100. Advertising inquiries from Europe and elsewhere should be addressed to Hilary Turnbull, imPRESS, 2 Penrith Avenue, Glasgow G46 6LU, UK; telephone +44.141.620.0106, fax: +44.141.620.0055. All advertisements are subject to approval by the Publisher.

Manuscripts should be directed to Arthur M. Krieg, the Editorial Office, Department of Internal Medicine, University of Iowa, 540 EMRB, Iowa City, IA 52242.

All authorized papers and editorial news and comments, opinions, findings, conclusions, or recommendations in *Antisense & Nucleic Acid Drug Development* are those of the author(s), and do not necessarily reflect the views of the journal and its publisher, nor does their publication in *Antisense & Nucleic Acid Drug Development* imply any endorsement.

Antisense & Nucleic Acid Drug Development is indexed in Index Medicus, Current Contents/Life Sciences, EMBASE, Biotechnology Citation Index, Science Citation Index, and SciSearch.

Antisense & Nucleic Acid Drug Development is a Journal Club selection.



# Case 1:21-cv-01015-JLH Document 174-2 Filed 03/20/23 Page 33 of 318 PageID #: AntiSense Nucleic Acid Drug Development

#### **Editors**

Arthur M. Krieg, M.D.
Department of Internal Medicine
University of Iowa
540 EMRB
Iowa City, Iowa 52242
(319) 335-6841
Fax: (319) 335-6887
E-mail: arthur-krieg@uiowa.edu

C.A. Stein, M.D., Ph.D.
Department of Medicine and
Pharmacology
Columbia University
College of Physicians and Surgeons
630 West 168th Street
New York, NY 10032
(212) 305-3606
Fax: (212) 305-7348

14 2'97

 $\hbox{$E$-mail: stein@cuccfa.ccc.columbia.edu}$ 

#### **Editorial Board**

Sudhir Agrawal
Shrewsbury, MA
Serge L. Beaucage
Bethesda

Frank Bennett

Carlsbad, CA

Lauren Black
Rockville, MD

Marvin H. Caruthers Boulder

Esther H. Chang Stanford

P. Dan Cook Carlsbad, CA

Stanley Crooke Carlsbad, CA

Fritz Eckstein
Göttingen, Germany

Joachim Engels Frankfurt

Robert P. Erickson Tucson

Sergio Ferrari Modena, Italy

Michael Gait Cambridge, UK

Wayne L. Gerlach
Canberra, Australia

Alan Gewirtz

Philadelphia

Donald Grierson

Loughborough, En

Loughborough, England

Sergei Gryaznov Foster City, CA

Claude Hélène Paris

William R. Hiatt Davis, CA

Jeffrey T. Holt Nashville

Jean-Louis Imbach Montpellier, France

Masayori Inouye Piscataway Patrick Iversen Omaha

Kuan-Teh Jeang Bethesda

Rudolph L. Juliano Chapel Hill

Ryszard Kole Chapel Hill

Bernard Lebleu

Montpellier, France

Lee Leserman Marseille

Robert L. Letsinger Evanston, IL

L. James Maher III Rochester, MN

Claude Malvy Villejuif, France

Dan Mercola San Diego

Paul S. Miller Baltimore

Ramaswamy Narayanan Nutley, NJ

L.M. Neckers
Bethesda

Wolfgang Nellen Kassel, Germany

Mike Nerenberg San Diego

Eiko Ohtsuka Sapporo, Japan

Robert Rando The Woodlands, TX

John C. Reed La Jolla

John J. Rossi Duarte, CA

Esther Saison-Behmoaras Paris Nava Sarver Bethesda Kevin Scanlon

Duarte, CA

Karl-Hermann Schlingensiepen Göttingen, Germany

Georg Sczakiel Heidelberg, Germany

Hartmut Seliger
Ülm, Germany
Zoe Shabarova
Moscow

Barbara Ramsey Shaw
Durham

Hermona Soreq Jerusalem Wojciech Stec Lodz, Poland

Martin Tabler
Heraklion/Crete, Greece

David M. Tidd Liverpool, UK

J.-J. Toulme Bordeaux

Paul Ts'o

Baltimore

Eugen Uhlmann Frankfurt

Valentin V. Vlassov Novosibirsk, Russia

Gerhart Wagner Uppsala, Sweden

Richard Wagner Foster City, CA

Daniel Weeks Iowa City

Eric Wickstrom

Philadelphia

Paul Zamecnik Shrewsbury, MA

Gerald Zon Hayward, CA

Founding Editor
James W. Hawkins

# Antisense & Nucleic Acid Drug Development

VOLUME /	NUMBER 3 JUNE	E 199
Original Articles  Rapid Measurement of Modified Oligon for Single-Stranded DNA. G.D. GRA	nucleotide Levels in Plasma Samples with a Fluorophore Specific Y and E. WICKSTROM	133
Tissue Distribution and Metabolism of the [32P]-Labeled Oligodeoxynucleoside Methylphosphonate-Neoglycopeptide Conjugate, [YEE(ah-GalNAc) <sub>3</sub> ]-SMCC-AET-pU <sup>m</sup> pT <sub>7</sub> , in the Mouse. J.J. HANGELAND J.E. FLESHER, S.F. DEAMOND, Y.C. LEE, P.O.P. TS'O, and J.J. FROST		
	sense Types: Morpholino, 2'-O-Methyl RNA, DNA, and FOSTER, SB. HUANG, D. WELLER, and J. SUMMERTON	151
In Vivo Metabolic Profile of a Phosphor C. CHAIX, and S. AGRAWAL	rothioate Oligodeoxyribonucleotide. J. TEMSAMANI, A. ROSKEY,	159
Identification of a Phosphodiester Hexanucleotide That Inhibits HIV-1 Infection <i>In Vitro</i> on Covalent Linkage of Its 5'-End with a Dimethoxytrityl Residue. H. FURUKAWA, K. MOMOTA, T. AGATSUN I. YAMAMOTO, S. KIMURA, and K. SHIMADA		, 167
Ferric Protoporphyrin IX (Heme). G.I	nan Hepatoma Cells Using Cationic Lipid Particles Conjugated to B. TAKLE, A.R. THIERRY, S.M. FLYNN, B. PENG, L. WHITE, A.R. GOLDBERG, and S.T. GEORGE	177
Review Article Morpholino Antisense Oligomers: Desig	gn, Preparation, and Properties. J. SUMMERTON and D. WELLER	187
Presentations from the First NIH Sym	nposium on Therapeutic Oligonucleotides	
Introduction. Y.S. CHO-CHUNG		197
Background of the Antisense Oligonucleotide Approach to Chemotherapy. P. ZAMECNIK		199
Recruiting the 2-5A System for Antisense Therapeutics. P.F. TORRENCE, W. XIAO, G. LI, H. CRAME M.R. PLAYER, and R.H. SILVERMAN		203
Controversies in the Cellular Pharmacole	ogy of Oligodeoxynucleotides. C.A. STEIN	207
Recombinational Repair of Genetic Mut	ations. A. COLE-STRAUSS, A. NÖE, and E.B. KMIEC	211
-	Gene-Based Therapeutic Approach. Y.S. CHO-CHUNG, N, K. NOGUCHI, R. SRIVASTAVA, and S. PEPE	217
Antisense c-myc Inhibition of Lymphom	na Growth. E. WICKSTROM	225
	*	

(continued)

## Case 1:21-cv-01015-JLH Document 174-2 Filed 03/20/23 Page 35 of 318 PageID #: 7136

Identification and Characterization of Second-Generation Antisense Oligonucleotides. N.M. DEAN and R.H. GRIFFEY	229
Protein Kinase C as a Target for Cancer Therapy. R.I. GLAZER	235
Pharmacology of Therapeutic Oligonucleotides. R.B. DIASIO and R. ZHANG	239
In Vivo Pharmacokinetics of Phosphorothioate Oligonucleotides Containing Contiguous Guanosines. S. AGRAWAL, W. TAN, Q. CAI, X. XIE, and R. ZHANG	
Ex Vivo Bone Marrow Purging with Oligonucleotides. R.C. BERGAN	251
Antisense Transforming Growth Factor- $\beta$ 1 in Wound Healing. HT. CHUNG, BM. CHOI, CD. JUN, SD. PARK, and JS. RIM	

Instructions for Authors

#### Review Article

# Morpholino Antisense Oligomers: Design, Preparation, and Properties

JAMES SUMMERTON and DWIGHT WELLER

#### **ABSTRACT**

Antisense promised major advances in treating a broad range of intractable diseases, but in recent years progress has been stymied by technical problems, most notably inadequate specificity, ineffective delivery into the proper subcellular compartment, and unpredictable activity within cells. Herein is an overview of the design, preparation, and properties of Morpholino oligos, a novel antisense structural type that solves the sequence specificity problem and provides high and predictable activity in cells. Morpholino oligos also exhibit little or no nonantisense activity, afford good water solubility, are immune to nucleases, and are designed to have low production costs.

#### INTRODUCTION

OLIGONUCLEOTIDES, OLIGONUCLEOTIDE ANALOGS, and other sequence-specific binding polymers designed to block translation of selected messenger RNAs (the sense strand) are commonly called "antisense oligos." Development of such oligos for therapeutic applications, which constitutes the epitome of rational drug design, entails selecting a target genetic sequence unique and critical to the pathogen or pathogenic state one wishes to treat. One then assembles an oligomer of genetic bases (adenine, cytosine, guanine, and thymine or uracil) complementary to that selected sequence. When such an antisense oligo binds to its targeted disease-causing sequence, it can inactivate that target and thereby alleviate the disease.

Antisense oligos offer the prospect of safe and effective therapeutics for a broad range of intractable diseases. Nonetheless, developing therapeutics that function by a true antisense mechanism presents a number of forbidding challenges. The oligos should achieve adequate efficacy at a concentration attainable within the cells of the patient. They should inhibit their selected target sequences without concomitant attack on any other sequences in the patient's pool of approximately 200 million bases of unique-sequence RNA. They should be stable in extracellular compartments and within cells. They must be deliverable into the cellular compartment(s) containing their targeted sequences. They should be adequately soluble in aqueous solution. They should exhibit little or no toxicity at therapeutic concentrations. Finally, they should be affordable, reflecting the in-

creasing awareness that health care, even for life-threatening conditions, should not expend an excessive portion of society's resources.

First-generation antisense oligos comprised natural genetic material (Belikova et al., 1967; Zamecnik and Stephenson, 1978; Summerton, 1979) and often contained crosslinking agents for binding their targets irreversibly (Summerton and Bartlett, 1978a,b). As the design challenges became more fully appreciated, a number of nonnatural antisense structural types (Fig. 1) were developed in an effort to improve efficacy, stability, and delivery. Of particular note are the early non-ionic DNA analogs developed by Miller and Ts'o, including phosphotriester-linked DNA (Miller, 1989a) and methylphosphonate-linked DNA (Miller, 1989b). Other nucleic acid analogs of note include carbamate-linked DNA (Stirchak et al., 1987), phosphorothioate-linked DNA (Stein and Cohen, 1989), phosphoroamidate-linked DNA (Froehler et al., 1988), α-DNA (Rayner et al., 1989), and 2'-O-methyl RNA (Shibahara et al., 1989). Figure 1B shows several novel antisense types that no longer resemble nucleic acids. These oligos contain acyclic backbone moieties, including nylon (Weller et al. 1991; Huang et al., 1991), the exceptionally high-affinity peptide nucleic acids (PNAs) (Egholm et al., 1992), and related types (Summerton and Weller, 1993a).

Although each of these newer structural types provides one or more significant advantages over the first-generation oligos, none yet appear to provide the full combination of properties needed in antisense therapeutics for clinical applications.

FIG. 1. Representative antisense structural types.

Herein we describe the design considerations used in developing a novel Morpholino structural type (Fig. 2), which affords antisense oligos having very high efficacy and specificity, immunity to nucleases, good aqueous solubility, and low production costs.

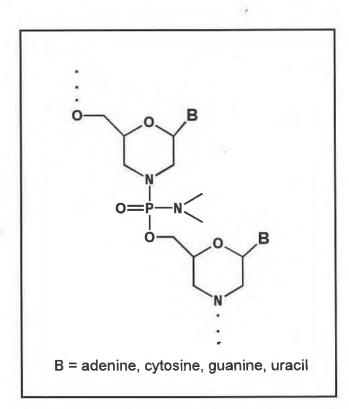


FIG. 2. Morpholino oligo structure.

### **DESIGN**

### Backbone structure

A dominant consideration in the design of most antisense oligos has been to devise a structure that provides resistance to nucleases while still resembling natural nucleic acids as closely as possible. This conservative approach has spawned a number of DNA analogs (Fig. 1A) that may be unduly expensive for routine applications requiring systemic delivery. The high cost of DNA and its analogs is due in part to the low abundance of DNA in production-scale source material and the difficulty in cleaving DNA to the deoxyribonucleosides required for preparing DNA analogs. An additional factor in their high cost is the complexity and expense of coupling to hydroxyls, required in forming the phosphoester intersubunit linkages of most DNA analogs.

Rather than trying to solve inherent cost problems after a structural type has been developed, a better approach is to incorporate fundamental cost advantages in the initial structural design stage. Following this strategy, we reasoned that more affordable antisense oligos might be possible if inexpensive ribonucleosides could be exploited as starting material. The order-of-magnitude cost advantage of ribonucleosides relative to deoxyribonucleosides (Summerton, 1992) derives from the sixfold greater abundance of RNA relative to DNA in production-scale source material (e.g., yeast cake) and the ease of cleaving

## Mase HOLINOV ANIOS ENSEMBLIGOS cument 174-2 Filed 03/20/23 Page 38 of 318 PageID #89

RNA to its component ribonucleosides. It is noteworthy that ribonucleosides are now directly available from special excreting strains of yeast, further reducing their cost. However, the use of ribonucleosides for preparation of RNA and RNA analogs presents two serious problems. First, during oligo assembly, one must selectively couple either the 2' or the 3' hydroxyl. This is typically achieved in a relatively expensive manner by selectively masking the 2' hydroxyl with a cleavable or noncleavable moiety. The second problem is that coupling to the 3' hydroxyl of the riboside is even more difficult and expensive than the corresponding coupling of deoxyribonucleosides.

We envisioned that these problems could be circumvented by converting the riboside moiety to a morpholine moiety (Stirchak et al., 1989; Summerton, 1990) (Fig. 3). Although oligomers assembled from such Morpholino subunits differ substantially from DNA, RNA, and analogs thereof, our initial modeling studies carried out in 1985 suggested that such novel Morpholino-based oligomers might constitute useful and highly cost-effective antisense agents. The simple and inexpensive ribose to morpholine conversion shown in Figure 3 replaces two poor nucleophiles (the 2' and 3' hydroxyls) with a single good nucleophile (the morpholine nitrogen) and allows oligo assembly via simple and efficient coupling to the morpholine nitrogen without the expensive catalysts and postcoupling oxidation steps required in the production of most DNA-like antisense oligos. It is noteworthy that in spite of the relatively low nucleophilicity of the morpholine nitrogen (p $K_a = 5.75$ ), we still typically achieve coupling efficiencies of 99.7% without using catalysts.

### Intersubunit linkage

We have assessed a substantial number of intersubunit linkage types, including the carbonyl, sulfonyl, and phosphoryl linkages (Fig. 4) (Summerton and Weller, 1991, 1993a,b; Stirchak et al., 1989). Although Morpholino oligos containing a number of such linkages provide effective binding to targeted genetic sequences, consideration of cost and ease of synthesis, chemical stability, aqueous solubility, and affinity and homogeneity of binding to RNA led us to focus on the phosphorodiamidate shown in Figure 2 as our principle linkage type for oligos targeted against single-stranded RNA sequences. These non-ionic phosphorodiamidate-linked Morpholino oligos exhibit quite good binding to complementary nucleic acids, particularly RNA sequences. Table 1 compares the temperature of melting  $(T_m)$  values at physiologic salt concentration for identical-sequence 20-mer oligos of three different antisense structural types paired with their complementary RNA. As seen in Table 1, RNA binding affinity is lowest for the phosphorothioate-linked DNA (S-DNA), appreciably higher for DNA, and highest for the Morpholino oligo.

### **PREPARATION**

Oligo assembly

Although phosphorodiamidate-linked Morpholino oligos can be assembled by a variety of methods, one relatively simple method that has proved effective (Summerton and Weller, 1993b) entails protection and activation of the Morpholino subunit (Fig. 5A). The activated subunits can be stored at low temperatures for many months without significant breakdown. Whereas they are relatively resistant to hydrolysis, they react rapidly ( $T_{1/2}$  of 1–2 minutes) with the morpholine nitrogen of growing chains on a 1% crosslinked polystyrene synthesis support loaded at 500  $\mu$ M/g of resin, with coupling efficiencies typically about 99.7%. A preferred oligo assembly cycle (Summerton and Weller, 1993b) is shown in Figure 5B. It is noteworthy that in large-scale syntheses, excess activated subunit used in the coupling step can be recovered and reused, effecting a further substantial reduction in production costs.

Because of cheaper starting materials and simpler, more efficient oligo assembly, we estimate that in large-scale production, the cost of these Morpholino antisense oligos will be at least an order of magnitude lower than the cost of corresponding DNA analogs (Summerton, 1992).

### **PROPERTIES**

Solubility

For an antisense oligo to have effective access to its target sequence within the cytoplasm of a cell, the oligo should show reasonable water solubility. Good water solubility may also prove essential for systemic delivery of antisense oligos. Conventional wisdom in the antisense field is that non-ionic antisense oligos invariably show poor water solubility. In this regard, it is interesting that a Morpholino dimer containing a rigid carbamate linkage shows little or no base stacking (Kang et al., 1992), and in the absence of special solubilizing groups, Morpholino oligomers containing such carbamate linkages are quite insoluble in aqueous solutions (Stirchak et al., 1989). In contrast, phosphorodiamidate-linked Morpholino oligos of the type shown in Figure 2 show excellent base stacking (Kang et al., 1992) and are several orders of magnitude more soluble in aqueous solutions. To illustrate the exceptional aqueous solu-

FIG. 3. Conversion of ribonucleoside to Morpholino subunit.

FIG. 4. Intersubunit linkage types for Morpholino oligos.

bility of Morpholino oligos of this type, we have dissolved 263 mg of a heteromeric 22-mer of the sequence 5'-GCUCGCA-GACUUGUUCCAUCAU in 1 ml of water (36 millimolal) at 20°C without reaching saturation.

We suggest that the poor water solubility of the carbamate-linked Morpholino oligos results at least in part from the difficulty of inserting the hydrophobic faces of the unstacked bases into an aqueous environment. In contrast, it seems likely that the excellent water solubility of the phosphorodiamidate-linked Morpholino oligos is a consequence of effective shielding of these hydrophobic faces from the polar solvent because of good stacking of the bases.

### Biologic stability

To achieve reasonable efficacy, an antisense oligo should not be degraded rapidly either extracellularly or within cells. In this regard, it has been demonstrated that DNA and 2'-O-methyl RNA are rapidly degraded and phosphorothioate DNA is slowly degraded by nucleases in blood and within cells (Hoke et al., 1991; Morvan et al., 1993). Although resistance to nucleolytic degradation can be improved by adding special groups to the termini (Cazenave et al., 1987) or by incorporating a few nuclease-resistant intersubunit linkages near each end (Larrouy et al., 1992), we believe a better solution, on the basis of both function and cost, is to use a backbone structure that is inherently immune to a broad range of degradative enzymes present in the blood and within cells. A further advantage of using a backbone structure that is not degraded in the body is that it avoids concerns that modified nucleosides or nucleotides resulting from degradation of an antisense oligo might be toxic or might be incorporated into cellular genetic material and thereby lead to mutations or other undesired biologic effects.

In experiments detailed elsewhere (Hudziak et al., 1996), it is demonstrated that Morpholino phosphorodiamidate oligos of

TABLE 1. MELTING TEMPERATURES OF RNA/OLIGO DUPLEXES

RNA/S-DNA	68.5°C
RNA/DNA	77.3°C
RNA/Morpholino	81.3°C

the type shown in Figure 2 are immune to a wide range of nucleases, including DNase I (an endonuclease that cleaves both single-stranded and double-stranded DNA), DNase II (cleaves between the 5' oxygen and the phosphorus of DNA linkages), RNase A (cleaves on the 3' side of pyrimidines), RNase T1 (cleaves on the 3' side of guanines), nuclease P1 (cleaves single-stranded RNA and DNA), phosphodiesterase (3' exonuclease for both RNA and DNA), Mung bean nuclease (cleaves single-stranded RNA and DNA), and benzonase (cleaves both single-stranded and double-stranded RNA and DNA, including linear, circular, and supercoiled). These Morpholino oligos have also been found to be immune to pronase E, proteinase K, and pig liver esterase, as well as degradative enzymes in serum and a liver homogenate.

### Antisense efficacy

Because of the excellent RNA binding affinity of oligos of this phosphorodiamidate-linked Morpholino structural type, it seemed likely Morpholino oligos would be effective in blocking translation of their targeted mRNAs, and this has been found to be the case. In cell-free translation experiments using a sensitive luciferase reporter, we have demonstrated that a Morpholino oligo 25 subunits in length, in both the presence and absence of RNase H, inhibits its targeted mRNA somewhat better than the corresponding S-DNA oligo in the presence of added RNase H, with both showing good efficacy at concentrations of 10 nM and above. Representative translational inhibition results are shown in Figure 6 (Summerton et al., 1997). A similar comparison of Morpholino and S-DNA antisense oligos targeted against murine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) mRNA in a cell-free translation system also showed greater activity for the Morpholino oligos (Taylor et al., 1996).

### Specificity

In the early days of antisense research, one of the most compelling arguments for antisense therapeutics was their promise of exquisite specificity for their targeted genetic sequences. However, as the most synthetically accessible antisense structural types (DNA and S-DNA) have come into broad use, it has become clear that these two structural types provide reasonable

FIG. 5. Protection, activation, and coupling of Morpholino subunits.

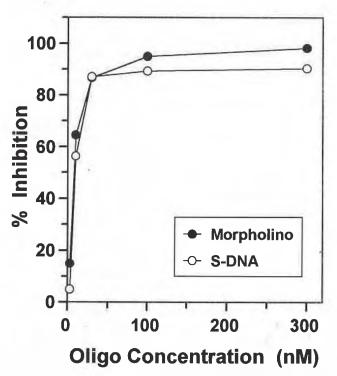


FIG. 6. Cell-free efficacy of Morpholino and S-DNA antisense oligos.

sequence specificity within only a very narrow concentration range (ANTIVIRALS Inc., 1993; Stein and Cheng, 1993).

We believe a key factor responsible for the low specificity of DNA and S-DNA oligos is their RNase H competency; that is, DNA and S-DNA form duplexes with complementary RNA that are readily cleaved by RNase H, an enzyme widely distributed in living organisms. The specificity problem arises because DNA/RNA and S-DNA/RNA duplexes as short as 5 base pairs in length are cleaved by RNase H (Crouch and Dirksen, 1982). Presuming about 6% of the genome is transcribed in higher animals, the patient's RNA pool will comprise about 200 million bases of unique-sequence RNA. With this level of sequence complexity, it is inevitable that antisense oligos will form many short transient duplexes with partially complementary nontarget sequences of inherent cellular RNAs. Cleavage of the RNA strand of such nontarget duplexes by endogenous RNase H (Larrouy et al., 1992; Cazenave et al., 1989) is expected to cause significant disruption of normal cellular translation. As this cleavage process releases the DNA or S-DNA in its original form, such oligos can continue the cycle of transiently pairing with additional nontarget cellular RNA sequences, cleavage of the RNA strand, and release of the antisense oligo. As a consequence, essentially every RNase H-competent oligo is expected to cleave hundreds to thousands of species of inherent cellular RNAs.

A second factor expected to contribute to superior specificity of Morpholino oligos relative to RNase H-competent types is that RNase H-independent oligos have far fewer potential targets in the inherent pool of cellular RNA. This is because most antisense structural types that do not support RNase H cleavage of their RNA targets have been found to be effective in blocking translation of their targeted mRNAs only when said oligos are complementary to sequences in the 5' leader region of that mRNA or when they are targeted against other special sites, such as splice junctions and transport signals [e.g., methylphosphonate DNA (Walder and Walder, 1988), α-DNA (Rayner et al., 1989), 2'-O-methyl RNA (Shibahara et al., 1989), and Morpholino (Summerton et al., 1997)]. We estimate that such special targetable regions constitute on the order of 2%-5% of the sequeces in the cellular RNA pool. Presumably, this targeting limitation reflects the ability of ribosomes to displace essentially all antisense oligos during translocation down the coding region of mRNAs.

Because an antisense oligo that does not support RNase H cleavage cannot effectively block functioning of an RNA when said oligo is bound to sequences outside of special targetable regions, such an oligo only needs to distinguish its target sequence from those 2%-5% of the cellular RNA sequences comprising special targetable regions. In contrast, antisense oligos that form RNase H-cleavable duplexes with RNA can be effective when targeted essentially anywhere along an RNA transcript (Walder and Walder, 1988), presumably because RNase H cleavage at the target site of the antisense oligomer destroys the RNA, rendering moot the oligo displacement capability of translocating ribosomes. Accordingly, RNase H-competent oligos (DNA and S-DNA) face the much greater specificity challenge of distinguishing selected target sequences from essentially the entire pool of cellular RNA sequences. As a consequence, RNase H-independent oligos, such as Morpholinos, should enjoy a 20-fold to 50-fold advantage in sequence specificity because of this more than order-of-magnitude reduction in the number of inherent nontarget cellular sequences of any given length that they can inhibit.

A third factor compromising the specificity of S-DNA oligos is their promiscuous binding to proteins (Krieg and Stein, 1995), including components of the cell's replication, transcription, and translation machinery.

Given these factors expected to limit the sequence specificity of RNase H-competent antisense structural types, particularly S-DNA, we set out to compare sequence specificities of S-DNA and Morpholino antisense oligos. To this end, we carried out stringent specificity assays in a cell-free translation system using two oligos of each structural type (Summerton et al., 1997). In these experiments, one oligo was perfectly complementary to its target mRNA to provide a measure of the total inhibition afforded by that oligo type. The other oligo incorporated 4 mispairs to that same mRNA target sequence, with the longest run of perfect pairing comprising 10 contiguous base pairs, to provide an estimate of the low-specificity component of the inhibition. The difference between these two inhibition values at each concentration than provided a measure of the high-specificity component, which we denote as "sequence-specific inhibition."

Figure 7 (experimental as in Summerton et al., 1997) shows that the S-DNA oligo achieved reasonable efficacy at concentrations above about 10 nM, but the sequence-specific component of its inhibition dropped below 50% at concentrations of only 100 nM and higher. The corresponding Morpholino oligo achieved even better efficacy at 10 nM while maintaining good sequence specificity through 10,000 nM, the highest concentration tested. Thus, in this stringent test of specificity, the Morpholino oligo achieved highly effective and specific antisense activity over a concentration range more than two orders of magnitude greater than the concentration range wherein the corresponding S-DNA achieved reasonable efficacy and specificity.

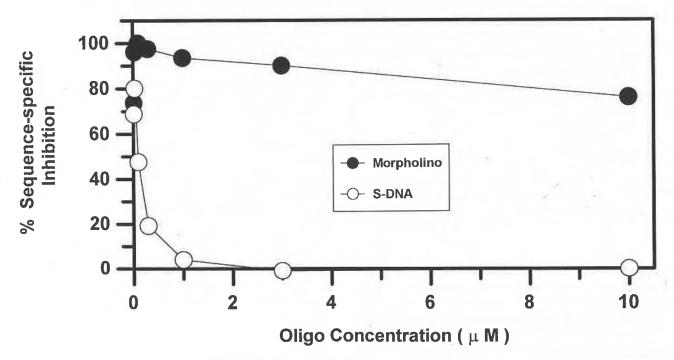


FIG. 7. Sequence specificity of Morpholino and S-DNA oligos.

Taylor et al., (1996) have reported that S-DNAs targeted against TNF- $\alpha$  mRNA showed very poor sequence specificity in a cell-free translation system, whereas the corresponding Morpholino oligos afforded good specificity over the full range tested.

### Activity in cells

For effective biologic activity, an antisense oligo must gain entry into the cellular compartments where the target genetic sequence is synthesized, processed, and functions-specifically, the cytosol/nuclear compartment. Our experiments with fluorescent-tagged Morpholino oligos suggested that these oligos enter mammalian cells by what appears to be endocytosis, but they do not appear to subsequently cross the endosomal or lysosomal membrane into the cytosol, based both on visualization of fluorescent-tagged oligos and a functional assay employing a transfected plasmid (Partridge et al., 1996). This result is in agreement with limitations on uptake of antisense oligos reported by others. Specifically, a number of studies have been reported that suggest that in the absence of experimental manipulations that compromise the cell membrane, both polyanionic oligos [e.g., S-DNA (Wagner et al., 1993; Tonkinson and Stein, 1994) and 2'-O-methyl RNA (Oberhauser and Wagner, 1992)] and non-ionic oligos [e.g., methylphosphonate DNA (Shoji et al., 1991) and PNAs (Bonham et al., 1995)] enter cells primarily or exclusively by endocytosis. Further, a number of studies on a variety of antisense structural types indicate that most or all of the antisense oligo that gains entry by endocytosis does not subsequently traverse

the endosomal or lysosomal membrane to enter in an intact form into the cytosol, where protein synthesis occurs (Oberhauser and Wagner, 1992; Shoji et al., 1991; Bonham et al., 1995).

However, we have found that antisense oligos can be easily delivered into cultured cells simply by passaging anchorage-dependent cells by the common procedure of scraping with a rubber policeman. This has been shown to achieve significant oligo entry into the cytosolic compartment if the oligo is present during the scraping (Partridge et al., 1996). Further, Morpholino oligos delivered into cells by such scrape loading show good activity and specificity therein, whereas corresponding S-DNA oligos (both antisense and control sequences) largely fail to inhibit their targets within scrape-loaded cells at concentrations up to 3  $\mu$ M in the medium and instead are often stimulatory (Summerton et al., 1997). Figure 8 shows a comparison of the activities of representative Morpholino and S-DNA oligos in scrape-loaded cells (experimental as in Summerton et al., 1997)

Taylor et al. (1996) have also compared the activity of S-DNA and Morpholino antisense oligos in cultured cells. In their studies, the S-DNAs were delivered into mouse macrophage-like cells (RAW 264.7) using lipofectin. Both oligo types were targeted against TNF- $\alpha$  mRNA, and treated cells were assessed for inhibition of lipopolysaccharide-induced TNF- $\alpha$  production. In agreement with our in-cell results, Taylor et al. report that both the antisense and control S-DNAs stimulated instead of inhibitied TNF- $\alpha$  production, whereas the Morpholino antisense oligo, although poorly delivered into the cells, afforded significant and specific inhibition of TNF- $\alpha$  production.

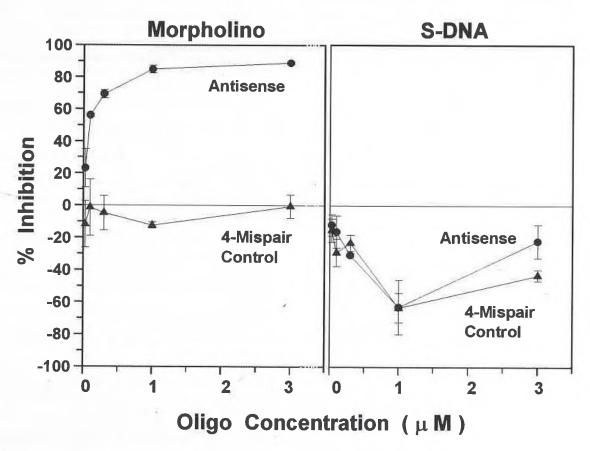


FIG. 8. In-cell activities of Morpholino and S-DNA oligos.

In vivo properties

To date, our principal efforts have focused on optimizing the Morpholino structural type and on studying the properties of Morpholino oligos at the biophysical level, in cell-free translation systems, and in cultured cells. In light of the promising results from those studies, we and several collaborators are now shifting our focus to *in vivo* studies.

A very preliminary ranging study was carried out to assess acute toxicity. In this study, a representative 20-mer Morpholino oligo in phosphate-buffered saline was injected intravenously into mice at doses ranging from 88 mg/kg to 700 mg/kg. No acute toxicity was seen at any of these doses. However, over a period of 2 weeks, an effect on body weight gain and ruffled coat was observed at the highest dose. Using the results from this ranging study, an extensive toxicity study has been initiated and will be the subject of a future report.

In addition, a variety of efficacy studies in mice and rats are in progress to assess the possible use of Morpholino oligos for therapeutic applications. We are also investigating possible methods for improving the delivery of these oligos into the cytosol/nuclear compartment of cells *in vivo*.

### DISCUSSION

Morpholino oligos meet key requirements for safe, effective, and affordable antisense therapeutics, including high efficacy at low nanomolar concentrations, high sequence specificity over a thousandfold concentration range, little or no nonantisense activity, total stability in blood and within cells, excellent water solubility, and low production costs relative to other antisense structural types. Our efforts are now focused on achieving effective delivery into the cytosol/nuclear compartment of cells by means suitable for therapeutic applications and on studying the activities of these oligos in animals.

### **ACKNOWLEDGMENTS**

We thank Donald Johnson, Christopher Matthews, Denis Burger, and Alan Timmins for helpful suggestions during the preparation of this manuscript.

### REFERENCES

- ANTIVIRALS Inc. Technical Report 3 (1993). Comparative studies of efficacy and specificity of translation inhibition in reticulocyte lysates. Antisense Res. Dev. 3, following p. 306.
- BELIKOVA, A., ZARYTOVA, V., and GRINEVA, N. (1967). Synthesis of ribonucleosides and diribonucleoside phosphates containing 2-chloroethylamine and nitrogen mustard residues. Tetrahedron Lett. 7, 3557–3562.
- BONHAM, M., BROWN, S., BOYD, A., BROWN, P., BRUCKEN-STEIN, D., HANVEY, J., THOMSON, S., PIPE, A., HASSMAN, F., BISI, J., FROEHLER, B., MATTEUCCI, M., WAGNER, R., NOBLE, S., and BABISS, L. (1995). An assessment of the antisense properties of RÑase H-competent and steric-blocking oligomers. Nucleic Acids Res. 23, 1197–1203.

- CAZENAVE, C., LOREAU, N., THUONG, N., TOULME, J., and HE-LEN, C. (1987). Enzymatic amplification of translation inhibition of rabbit beta-globin mRNA mediated by antimessenger oligodeoxynucleotides covalently linked to intercalating agents. Nucleic Acids Res. 15, 4717–4736.
- CAZENAVE, C., STEIN, C., LOREAU, N., THUONG, N., NECK-ERS, L., SUBASINGHE, C., HELENE, C., COHEN, J., and TOULME, J. (1989). Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides. Nucleic Acids Res. 17, 4255.
- CROUCH, R., and DIRKSEN, M. (1982). Ribonuclease H. In: *Nucleases*. S. Linn and R. Roberts, eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), pp. 211–241.
- EGHOLM, M., BUCHARDT, O., NIELSEN, P., and BURG, R. (1992). Peptide nucleic acids (PNA). Oligonucleotide analogues with an achiral peptide backbone. J. Am. Chem. Soc. 114, 1895–1897.
- FROEHLER, B., NG, P., and MATTEUCCI, M. (1988). Phosphoramidate analogues of DNA: Synthesis and thermal stability of heteroduplexes. Nucleic Acids Res. 16, 4831–4839.
- HOKE, G., DRAPER, K., FRIER, S., GONZALEZ, C., DRIVER, V., ZOUNES, M., and ECKER, D. (1991). Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization and antiviral efficacy versus herpes simplex virus infection. Nucleic Acids Res. 19, 5743–5748.
- HUANG, S., NELSON, J., and WELLER, D. (1991). Acyclic nucleic acid analogs: Synthesis and oligomerization of gamma,4-diamino-2-oxo-1(2H)-pyrimidinepentanoic acid and sigma,4-diamino-2-oxo-1(2H)-pyrimidinehexanoic acid. J. Org. Chem. 56, 6007–6018.
- HUDZIAK, R., BAROFSKY, L., BAROFSKY, D., WELLER, D.L., HUANG, S-B., and WELLER, D.D. (1996). Resistance of Morpholino phosphorodiamidate oligomers to enzymatic degradation. Antisense Nucleic Acid Drug Dev. 6, 267–272.
- KANG, H., CHOU, P., JOHNSON, C., WELLER, D., HUANG, S., and SUMMERTON, J. (1992). Stacking interactions of ApA analogues with modified backbones. Biopolymers 32, 1351–1363.
- KRIEG, A., and STEIN, C. (1995). Phosphorothioate oligodeoxynucleotides: Antisense or anti-protein? Antisense Res. Dev. 5, 241.
- LARROUY, B., BLONSKI, C., BOIZIAU, C., STUER, M., MOREAU, S., SHIRE, D., and TOULME, J. (1992). RNase H-mediated inhibition of translation by antisense oligodeoxyribonucletides; Use of backbone modification to improve specificity. Gene 121, 189–194.
- MILLER, P. (1989a). Non-ionic antisense oligonucleotides: Oligonucleotide alkylphosphotriesters. In: Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression. J. Cohen, ed. (CRC Press, Inc., Boca Raton, FL), pp. 82–85.
- MILLER, P. (1989b). Non-ionic antisense olgionucleotides: Oligonucleotides methylphosphonates. In: Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression. J. Cohen, ed. (CRC Press, Inc., Boca Raton, FL), pp. 85–92.
- MORVAN, F., PORUMB, H., DEGOLS, G., LEFEBVRE, I., POM-PON, A., SPROAT, B., RAYNER, B., MALVY, C., LEBLEU, B., and IMBACH, J. (1993). Comparative evaluation of seven oligonucleotide analogues as potential antisense agents. J. Med. Chem. 36, 280–287.
- OBERHAUSER, B., and WAGNER, E. (1992). Effective incorporation of 2'-O-methyl-oligoribonucleotides into liposomes and enhanced cell association through modification with thiocholesterol. Nucleic Acids Res. 20, 533–538.
- PARTRIDGE, M., VINCENT, A., MATTHEWS, P., PUMA, J., STEIN, D., and SUMMERTON, J. (1996). A simple method for delivering Morpholino antisense oligos into the cytoplasm of cells. Antisense Nucleic Acids Drug Dev. 6, 169–175.
- RAYNER, B., MALVY, C., PAOLETTI, J., LEBLEU, B., PAOLETTI, C., and IMBACH, J. (1989).  $\alpha$ -Oligodeoxynucleotide ana-

- logues. In: Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression. J. Cohen, ed. (CRC Press, Inc., Boca Raton, FL), pp. 119–136.
- SHIBAHARA, S., MUKAI, S., MORISAWA, H., NAKASHIMA, H., KOBAYASHI, S., and YAMAMOTO, N. (1989). Inhibition of human immunodeficiency virus (HIV-1) replication by synthetic oligo-RNA derivatives. Nucleic Acids Res. 17, 239–252.
- SHOJI, Y., AKHTAR, S., PERIASAMY, A., HERMAN, B., and JU-LIANO, R. (1991). Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. Nucleic Acids Res. 19, 5543–5550.
- STEIN, C., and CHENG, Y. (1993). Antisense oligonucleotides as therapeutic agents—Is the bullet really magical? Science **261**, 1004–1012.
- STEIN, C., and COHEN, J. (1989). Phosphorothicate oligonucleotide analogs. In: *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*. J. Cohen, ed. (CRC Press, Inc., Boca Raton, FL), pp. 97–117.
- STIRCHAK, E., SUMMERTON, J., and WELLER, D. (1987). Uncharged stereoregular nucleic acid analogues. 1. Synthesis of a cytosine-containing oligomer with carbamate internucleoside linkages. J. Org. Chem. 52, 4202–4206.
- STIRCHAK, E., SUMMERTON, J., and WELLER, D. (1989). Uncharged stereoregular nucleic acid analogs: 2. Morpholino nucleoside oligomers with carbamate internucleoside linkages. Nucleic Acids Res. 17, 6129–6141.
- SUMMERTON, J. (submitted in 1973, published in 1979). Intracellular inactivation of specific nucleotide sequences: A general approach to the treatment of viral diseases and virally mediated cancers. J. Theor. Biol. 78, 77–99.
- SUMMERTON, J. (1990). Polynucleotide assay reagent and method. Canadian Patent 1,268,404.
- SUMMERTON, J. (1992). Cost-effective antisense structures. In: *Biotechnology International*. (Century Press, London), pp. 73–77.
- SUMMERTON, J., and BARTLETT, P. (1978a). Sequence-specific crosslinking agents for nucleic acids. Use of 6-bromo-5,5-dimethoxyhexanohydrazide for crosslinking cytidine to guanosine and crosslinking RNA to complementary sequences of DNA. J. Mol. Biol. 122, 145–162.
- SUMMERTON, J., and BARTLETT, P. (1978b). Nucleic acid crosslinking agent and affinity inactivation of nucleic acids therewith. US Patent 4,123,610.

- SUMMERTON, J., STEIN, D., HUANG, S., MATTHEWS, P., WELLER, D., and PARTRIDGE, M. (1997). Morpholino and phosphorothioate antisense oligomers compared in cell-free and in-cell systems. Antisense Nucleic Acids Drug Dev. 7, 63–70.
- SUMMERTON, J., and WELLER, D. (1991). Uncharged morpholinobased polymers having achiral intersubunit linkages. US Patent 5.034.506.
- SUMMERTON, J., and WELLER, D. (1993a). Polynucleotide assay reagent and method. US Patent 5,217,866.
- SUMMERTON, J., and WELLER, D. (1993b). Uncharged Morpholino-based polymers having phosphorus containing chiral intersubunit linkages. US Patent 5,185,444.
- TAYLOR, M., PAULAUSKIS, J., WELLER, D., and KOBZIK, L. (1996). *In vitro* efficacy of Morpholino-modified antisense oligomers directed against tumor necrosis factor- $\alpha$  mRNA. J. Biol. Chem. **22**, 17445–17452.
- TONKINSON, J., and STEIN, C. (1994). Patterns of intracellular compartmentalization, trafficking and acidification of 5'-fluorescein-labeled phosphodiester and phosphorothioate oligodeoxynucleotides in HL60 cells. Nucleic Acids Res. 22, 4268–4275.
- WAGNER, R., MATTEUCCI, M., LEWIS, J. GUTIERREZ, A., MOULDS, C., and FROEHLER, B. (1993). Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. Science **260**, 1510–1513.
- WALDER, R., and WALDER, J. (1988). Role of RNase H in hybrid-arrested translation by antisense oligonucleotides. Proc. Natl. Acad. Sci. USA 85, 5011–5015.
- WELLER, D., DALY, D., OLSON, W., and SUMMERTON, J. (1991).

  Molecular modeling of acyclic polyamide oligonucleotide analogues. J. Org. Chem. 56, 6000–6006.
- ZAMECNIK, P., and STEPHENSON, M. (1978). Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc. Natl. Acad. Sci. USA 75, 280–284.

Address reprint requests to:

James Summerton, Ph.D.

ANTIVIRALS Inc.
4575 S.W. Research Way, Suite 200

Corvallis, OR 97333

Received January 28, 1997; accepted in revised form March 6, 1997

# EXHIBIT 9

215

### Morpholinos and PNAs compared

### James E. Summerton

President, Gene Tools, Inc. One Summerton Way, Philomath, OR 97370, U.S.A. (e-mail: jsummerton@gene-tools.com)

### Introduction

This chapter will compare and contrast the properties and applications of two leading antisense molecules, Peptide Nucleic Acids (PNAs)<sup>1,2</sup> and Morpholinos<sup>3,4</sup>. Where appropriate, I discuss the compelling advantages which these two advanced 'blocker' types provide relative to 'modifier' types of antisense molecules. Some of the properties which are compared include chemical synthesis strategies, chemical stabilities, backbone flexibilities, aqueous solubilities, target selection criteria, target binding affinities, and sequence specificities.

Since the mid-1980s, phosphorothioate-linked DNA oligos (S-DNAs) have dominated the antisense field. For many antisense applications, however, advanced non-ionic oligos provide a far better combination of properties, including stability in biological systems, high efficacy and specificity, lack of toxicity, and freedom from non-antisense effects. Prior to the discovery of PNAs by Nielsen et al., I was the first person to conceptualize and to synthesize morpholinos, recognizing their advantages for antisense chemistry. Morpholinos and PNAs share a number of key properties, such as non-ionic backbones whose structures differ radically from that of nucleic acids, resistance to enzymatic degradation, and high (Morpholinos) or very high (PNAs) affinity for complementary RNA sequences. In the context of diagnostics, a particularly valuable property of both structural types is that they strongly pair to complementary genetic sequences under conditions which disrupt secondary structures of nucleic acids. Another particular advantage of morpholinos, and part of the original impetus to develop them, is the fact that they are relatively cheap to produce; the subunits of Morpholinos can be assembled into antisense oligos via simple and efficient coupling to the morpholine nitrogen, without the expensive catalysts and post-coupling oxidation steps required in the production of most nucleic acid analogs.

In spite of their many similarities, Morpholinos and PNAs also exhibit significant differences which translate to differing advantages in particular applications. Two key differences which bear on their preferred applications are: 1) PNAs have higher affinities for RNA than do Morpholinos, though both structural types form duplexes with RNA which are more stable than DNA/RNA duplexes, and much more stable than S-DNA/RNA duplexes; 2) Morpholinos are highly soluble in aqueous solutions, generally 5 to 30 mM for 25-mers, depending on sequence, whereas PNAs are typically several hundred-fold less soluble. As a consequence of these differing properties, it appears that PNAs are better suited for high-affinity applications such as targeting short sequences (e.g., the exposed segment of telomerase RNA) and for discriminating between single base differences, as in SNPs (single nucleotide polymorphisms). Conversely, Morpholinos excel in applications which require high aqueous solubility and exquisite discrimination between a targeted mRNA and tens of thousands of non-target mRNAs, such as in vivo applications with developing embryos and other complex systems.

### Classification of antisense structural types

Sequence-specific nucleic acid-binding oligomers ('oligos') can be divided into two categories:

- 1) 'blocker' oligos which hydrogen bond to, but do not modify their complementary (targeted) genetic sequences;
- 'modifier' oligos which H-bond to and then modify their targeted sequences, either directly by crosslinking or cleavage, or indirectly via RNase H-mediated degradation.

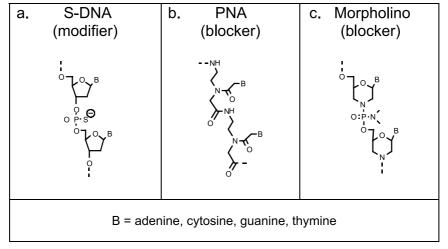


Figure 1. Representative oligo structural types.

Oligos in these two categories differ fundamentally in regard to which sequences they can target in an RNA transcript. Specifically, modifier oligos, exemplified by S-DNAs (Figure 1a), have the potential to target sites anywhere in an RNA transcript. In contrast, blocker oligos, exemplified by PNAs (Figure 1b) and Morpholinos (Figure 1c), are generally ineffective when targeted against intron sequences in a pre-mRNA, unless the target site is immediately adjacent to a splice site. Blocker oligos are also generally ineffective when targeted against amino acid-coding sequences more than about 40 bases 3' to the AUG translational start site in an mRNA. This is probably because once a ribosome completes its assembly at the AUG translational start site, its ATP-dependent unwinding activity becomes capable of displacing nearly all blocker oligos from their targeted sites in the downstream amino acid-coding region. 1,4,6

Figure 2a illustrates the limited regions of an RNA transcript which are potentially available for targeting by blocker oligos, currently estimated at about 5% of the total length of the average RNA transcript. Figure 2b illustrates the extensive region of an RNA transcript which is potentially available for targeting by modifier oligos of the types which effect cleavage or degradation of their targeted sequences, essentially 100% of RNA transcripts.

From a targeting perspective, at first glance the modifier oligos (e.g., S-DNAs) appear attractive because they offer the possibility of targeting sites throughout an RNA transcript (Figure 2b). However, in practice this apparent advantage is greatly lessened because selecting effective targets for modifier oligos

is often highly unpredictable and requires much empirical experimentation, <sup>7,8</sup> presumably because most possible target sites are unavailable due to secondary structures in the RNA transcripts and/or due to other poorly understood factors.

In contrast to the case for modifier oligos, advanced types of blocker oligos (PNAs and Morpholinos) targeted against sequences within their targetable regions (Figure 2a) generally give predictable and effective results. This is probably because the high RNA-binding affinities of advanced blocker types allow them to efficiently invade the extensive RNA secondary structures common to natural RNAs.

### Preparation of morpholinos and PNAs

Subunit synthesis

In the context of custom oligos for research applications, the costs of starting materials, ease and yield of key steps in subunit syntheses, and the costs of oligo assembly and processing are of lesser interest to both producers and users because labor expenses generally dominate the cost of custom oligos. However, for larger-scale applications, such as clinical diagnostics and therapeutics, these factors play a major role in the cost of the finished oligo. Because production costs will be an important factor in clinical applications, these costs will be discussed below.

PNA subunits are prepared from the standard purine (A and G) and pyrimidine (C and T) nucleobases. The key step in producing PNA subunits is selective alkylation on the nitrogen at the 9 position of the purines

and the 1 position of the pyrimidines, as illustrated in Figure 3a. Any lack of selectivity in this alkylation reaction will require careful purification of the desired product. In contrast to alkylation of the bases, adding the N-protected aminoethyl glycine backbone moiety and adding appropriate nucleobase protective groups should be relatively straightforward and should give good yields and purities.

Morpholino subunits are prepared from the natural rA, rC, and rG ribonucleosides. For the fourth subunit, we prefer to use synthetic rT instead of the natural rU because of the positive impact T bases have on RNA-binding affinities of the resulting oligos. The key steps in synthesis of Morpholino subunits are as follows: oxidative opening of the 5-membered ribose ring; closing the resulting dialdehyde on ammonia to give a 6-membered morpholine ring; and reductive removal of the original 2' and 3' hydroxyls, as illustrated in Figure 3b. These three steps are carried out sequentially in a single pot without intervening purifications. Once the ribose-to-morpholine conversion is accomplished, adding appropriate protective groups and adding the chlorophosphoroamidate moiety to the original 5' oxygen are relatively straightforward and give good yields and purities. It is noteworthy that in the dry state the final protected/activated Morpholino subunits are stable for many months at -20°C.

Table 1 shows approximate relative costs of the key subunit starting materials for PNA and Morpholino subunits from representative chemical supply companies, and for comparison, subunits for DNA analogs such as S-DNAs.

Both PNAs and Morpholinos enjoy a significant advantage over DNA analogs in regard to cost of starting materials, although some of this cost advantage is lessened due to the costs and yield losses in converting the nucleobases and ribonucleosides to their respective PNA and Morpholino subunit structures.

### Oligo assembly

In regard to assembling subunits into oligos, PNAs are typically assembled using a 3-reaction cycle: coupling, capping, and deprotection, as shown in Figure 4a. Including the intervening washes, the subunit addition cycle typically consists of 7 to 9 individual steps. 10,11,12

For assembly of Morpholinos, we use a very simple 2-reaction subunit addition cycle analogous to that used for active-ester peptide synthesis. The two reactions are coupling and deprotection, as shown in

Figure 4b. A capping step is not used because coupling and deprotection efficiencies are very high (estimated at about 99.7% for each reaction). Our newest subunit addition cycle for Morpholino oligos comprises these two reaction steps plus three wash steps, for a frugal 5-step assembly cycle carried out on a simple custom-made synthesizer. Through careful selection of solvent combinations, about 80% of the expensive solvent components can be recovered and, after distillation, reused. This recycling allows for cost saving on both the purchase of more solvents and disposal of used solvents. In principle, because of the good stability of the activated Morpholino subunits, much of the excess subunit used to drive the coupling to completion can also be recovered and reused. However, this is only practical in large-scale synthesis of an individual oligo. The recovery and reuse of both subunits and solvents has the potential to substantially reduce oligo costs in large-scale production of Morpholino therapeutics.

### Properties of morpholinos and PNAs

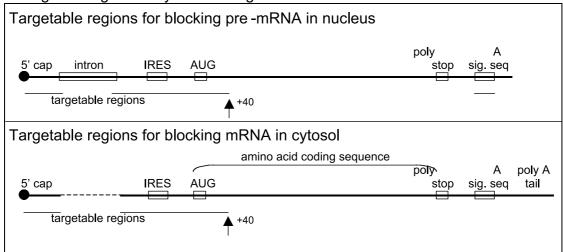
Chemical stability

The PNA backbone is stable to strong bases (which would degrade RNA) and to strong acids (which would depurinate DNA). The only significant instability of a PNA chain is when it has a free aminoethyl N-terminus, which occurs briefly during each coupling cycle in oligo assembly  $^{10}$  and would occur if one failed to cap the N-terminus on completion of the oligo. Because of favorable geometry, this aminoethyl moiety (pKa  $\sim 10.5$ ) can cause rearrangements and subunit deletions.  $^{10}$  Capping or otherwise modifying the N-terminus after completion of oligo assembly effectively stabilizes the finished oligo.

A particular advantage arising from the exceptionally high chemical stability of the PNA backbone is that while the oligo is still on the synthesis resin, a series of amino acids can be added to the oligo to generate a peptide component suitable for enhancing delivery into cells, or suitable for diagnostic applications. The PNA component easily survives the rather harsh conditions required for removal of protective groups on the side chains of that added peptide.

The Morpholino backbone is also stable to strong bases, but in contrast to the acid stability of PNAs, the Morpholino backbone is cleaved by strong acids, such as trifluoroacetic acid. While the sensitivity of Morpholinos to strong acids does impose some limitations

### a. Regions targetable by blocker oligos



### b. Regions targetable by modifier oligos

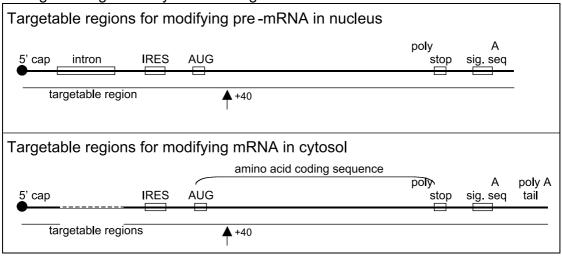


Figure 2. Targetable regions in RNA transcripts.

Table 1. Relative costs of starting materials

Oligo type	Starting material	Approximate relative cost per mole
PNA	Nucleobases	1
Morpholino	Ribonucleosides	2
DNA analogs	Deoxyribonucleosides	70

Figure 3. Key steps in subunit synthesis.

on possible chemical manipulations, that same sensitivity also affords at least one significant advantage – a fast and easy way to confirm sequence. In this method, a small portion of synthesis resin with completed oligo still attached is treated with neat TFA (40 minutes at room temperature) to generate on average of one cleavage per oligo chain. The resin is then washed free of TFA and 3′-terminal oligo fragments, and added to concentrated ammonium hydroxide to cleave the resin-bound 5′-terminal oligo fragments from the resin and to deprotect the nucleobases. A mass spectrum directly provides that oligo's sequence simply by tabulating the mass differentials between consecutive fragment peaks.

### Biological stability

A major challenge in using antisense oligos has been retention of the integrity of the oligo for a sufficient length of time to achieve the desired experimental or therapeutic effect. For example, bare RNA and DNA oligos introduced into cells undergo substantial enzymatic degradation in a matter of minutes. Various modifications of DNA and RNA backbones (e.g., replacing a sp. pendant oxygen on the phosphorous with a sulfur, as in S-DNAs, or adding an alkyl group to

the 2' oxygen of RNA, as in 2'O-Methyl RNA) as well as modifying the termini of the oligos to block exonucleases<sup>33</sup> all serve to improve stabilities in biological systems. Nonetheless, for close analogs of DNA and RNA having anionic intersubunit linkages, enzymatic degradation generally remains a significant problem in longer-term experiments.

Unlike the biological instabilities of close analogs of RNA and DNA, the radical design departure from natural nucleic acid structure embodied in PNAs and Morpholinos, coupled with their non-ionic intersubunit linkages, renders them highly resistant to enzymatic degradation in blood and within cells. <sup>14,15</sup> This stability to enzymatic degradation provides a compelling advantage in applications requiring long-term activity in biological systems, such as in studies in embryos <sup>16</sup> and in therapeutics.

### Backbone flexibility

A significant difference between PNAs and Morpholinos is that PNAs have significantly greater backbone flexibility. Specifically, as illustrated by the acyclic conformation on the left side of Figure 5a, PNAs have 7 bonds per subunit which can undergo relatively free rotations, though this may be transiently reduced to

5 freely rotatable bonds for some fraction of the subunits due to intramolecular H-bonding, as illustrated by the pseudo-ring conformation on the right side of Figure 5a. Molecular modeling suggests that the more flexible backbone of PNAs should favor applications such as triplex formation, including PNA/DNA/DNA and PNA/RNA/PNA triplexes. Indeed, PNAs are well known for excelling in such triplex applications.<sup>17,18</sup>

In contrast to the relatively flexible backbone of PNAs, Morpholinos have a more rigid backbone because they have only 4 bonds per subunit which can undergo relatively free rotations, as illustrated in Figure 5b.

We have found that a key requirement for achieving good antisense activity with high-affinity nonionic oligos is that they must not contain undue self-pairing potential. Self-pairing is a particular problem for PNAs and to a lesser extent, Morpholinos, because in contrast to the case for oligos having ionic backbones, with non-ionic oligos there is no electrostatic repulsion between the backbones of paired segments to help counterbalance the Watson/Crick pairing. Consequently, when significant self-pairing potential is present in such an oligo, self-pairing is likely to dominate to the exclusion of the desired oligo/target pairing.

In the context of relative backbone rigidities, a likely consequence of the greater flexibility of the PNA backbone, compared to the more rigid Morpholino backbone, is that the PNA oligo can more readily adopt a conformation suitable for self-pairing. To put this in semi-quantitative terms, from our experience in targeting many antisense oligos, it appears that the more flexible PNAs can have no more than about 8 contiguous Watson/Crick H-bonds of self-pairing before they start to suffer a reduction in antisense efficacy. In contrast, the more rigid Morpholinos can have up to about 11 contiguous Watson/Crick H-bonds of self-pairing without suffering significant loss of antisense activity. It should be noted that these numbers of allowable H-bonds are estimates, but may vary up or down by about one H-bond for particular sequences. Table 2 illustrates representative sequences having the approximate maximum amounts of self-pairing which appear to still allow good antisense activity for each of the structural types. Note that an A/T or A/U pair contributes 2 Watson/Crick H-bonds, while a G/C pair contributes 3 Watson/Crick H-bonds.

This modest difference in allowable amount of self-pairing potential for the two structural types results in considerable latitude in selecting effective tar-

Table 2. Approximate maximum allowable amounts of self-pairing

PNA:	-GCA-	-ATTA-
	-CGT-	-TAAT-
Morpholino:	-GCAC-	-ATTAC-
	-CGTG-	-TAATG-

gets for Morpholino oligos<sup>19</sup> as compared to rather severe restrictions imposed in selecting effective targets for PNAs.<sup>20</sup> The greater latitude in picking Morpholino targets is particularly advantageous when one wishes to target a relatively long RNA sequence (20 to 30 bases) in order to maximize the chance of complementing a suitable single-stranded region of the RNA, which appears to be needed for efficient initiation of oligo/target pairing.

### Aqueous solubility

Conventional wisdom has long held that oligos having non-ionic backbone structures invariably exhibit poor aqueous solubility. Indeed, until recently there was much support for this view, evidenced by the poor aqueous solubilities of the multiple non-ionic structural types shown in Figure 6. Solubilities of these structural types typically are limited to only about 10 to 100  $\mu$ M, depending on length and sequence.  $^{21,22,23,24,25}$ 

While a variety of solubilizing moieties have been added to these oligos to improve their limited aqueous solubilities (e.g., terminal phosphate on Methylphosphonates, polyethylene glycol on DNA carbamates and Morpholino carbamates, one or more lysines on PNAs), nonetheless, the inherent low solubilities of the core oligos often leads to aggregation and precipitation in many biological applications.

Surprisingly, non-ionic Morpholino oligos with freely rotatable bonds in the intersubunit linkage have been found to have excellent water solubility. To illustrate the importance of freely rotatable bonds, at 37 °C carbamates are known to exhibit restricted rotation, while sulfamides and phosphoroamidates are known to exhibit relatively free rotation. In this context, we have found that a Morpholino oligo having the more rigid carbamate intersubunit linkages (Figure 7a) is several hundred fold less water soluble than a corresponding Morpholino oligo containing the more flexible sulfamide or phosphoroamidate intersubunit linkages (Figures 7b–c).

221

The likely reason for this great difference in water solubility between these Morpholino subtypes is that the restricted rotation of the carbamate linkage largely prevents stacking of the bases, <sup>26</sup> so that dissolution in an aqueous environment then requires an energetically unfavorable insertion of the hydrophobic faces of the unstacked bases into water. Conversely, molecular modeling suggests that the free rotations of the phosphorodiamidate and sulfamide linkages should allow excellent stacking of the bases, and this has been confirmed experimentally for the phosphorodiamidate linkage. <sup>26</sup> This base stacking translates into excellent water solubility, presumably because the stacking effectively hides the hydrophobic faces of the bases from the aqueous environment.

One practical consequence of the excellent aqueous solubility of phosphorodiamidate-linked Morpholinos (typically 5 to 30 milliMolar for 25-mers) is that a minimal volume of a highly concentrated oligo solution can be injected into quite small eggs or early-stage embryos (e.g., zebrafish), as is required for developmental studies. This high water solubility, combined with exquisite sequence specificity, negligible toxicity, lack of non-antisense effects, and stability in biological systems, have made Morpholino oligos the preferred tools for selective gene knockdown studies in developmental biology. 9.27

### Salt dependence of binding

An important consequence of an oligo having a nonionic backbone, as is the case for PNAs and Morpholinos, is that their binding affinity for complementary genetic sequences is relatively insensitive to the ionic strength of the medium. Figure 8a compares  $T_m$  (melting temperature) values for 20-mer DNA/DNA and corresponding Morpholino/DNA duplexes as a function of salt concentration. Figure 8b compares  $T_m$  values for 20-mer DNA/RNA and corresponding Morpholino/RNA duplexes as a function of salt concentration.

PNA/DNA and PNA/RNA duplexes also exhibit similar independence between  $T_{\rm m}$  and salt concentration.  $^{1}$ 

As will be described in the section below on 'applications', this independence between  $T_m$  and salt concentration provides a dramatic advantage over classical anionic DNA and RNA oligomers and polymers in probe diagnostic applications.

RNA-binding affinity

Blocker-type oligos must tightly bind to their targeted RNA sequences in order to prevent RNA processing (e.g., splicing), readout (translation), or other functions (e.g., extension of telomers) of their targeted RNA transcripts. Figure 9 shows thermal transitions of various oligo/RNA duplexes (20-mers) at physiological salt concentration.

For comparison, we have found that at physiological salt concentration, PNA/RNA duplexes typically have thermal transitions similar to that of 2'O-Methyl RNA/RNA duplexes.

From Figure 9, one can see that Morpholino/RNA duplexes are more stable than corresponding DNA/RNA duplexes, and much more stable than corresponding S-DNA/RNA duplexes. While not included in Figure 9, under the same conditions we have found that PNA/RNA duplexes typically have substantially greater stability (about 8° to 10°C for 20-mers) than the already high stability of Morpholino/RNA duplexes.

It has been postulated that it is their high affinity for RNA which allows Morpholinos, and probably PNAs as well, to efficiently invade even quite stable RNA secondary structures,4 while the much lower affinity of S-DNAs (cf. Figure 9) necessitates an exhausting search for suitable RNA target sequences.<sup>7,8</sup> Such searches are probably necessarily extensive because in natural RNAs unstructured regions and regions having secondary structures of only minimal stability are relatively rare. Thus, the widely differing RNA-binding affinities between S-DNAs and the advanced non-ionic Morpholinos and PNAs may account for why Morpholinos, and probably PNAs, typically have an exceptionally high targeting success rate<sup>9</sup> (on the order of 75% to 85%), while S-DNAs generally have a much poorer targeting success rate<sup>7,8</sup> (on the order of 10%).

### Minimum Inactivating Length (MIL)

A useful measure of an antisense structural type is its 'Minimum Inactivating Length' (MIL), which may be defined as the shortest length of oligo of a given structural type which achieves substantial inhibition of its targeted sequence at a concentration typically achievable within cells. It should be noted that the measured MIL value for a given structural type varies somewhat as a function of sequence, G+C content, and concentration of the oligos tested. Nonetheless, by testing a range of oligo lengths targeted against the same target

222

Table 3. MIL values for different structural types

Structural type	MIL value
S-DNA	8
PNA	10
Morpholino	15

region, one can obtain reasonable comparative MIL values for various structural types of interest.<sup>4</sup>

Figure 10 shows the results of experiments carried out to estimate MIL values of S-DNAs, PNAs, and Morpholinos in a cell-free translation system (with added RNase H to afford good S-DNA activities). In these activity-versus-length experiments a set of oligos of the three structural types ranging in length from 8 bases to 30 bases were targeted against a region of rabbit alpha-globin leader sequence (Figure 10a), and a second set were targeted against a region of the Hepatitis B virus (HBV) leader sequence (Figure 10b). These two sets of oligos at a concentration of 300nM were assessed in a cell-free translation system for their abilities to inhibit translation of a downstream luciferase-coding sequence. The experimental procedures, oligo sequences, and RNA targets used in this study are detailed elsewhere.<sup>4,28</sup>

Table 3 gives the approximate MIL values derived from this length-versus-activity study.

As will be discussed in the next section, these differing MIL values strongly influence the preferred applications of PNAs and Morpholinos, particularly in regard to applications in complex systems.

### Specificity

The first strategy as regards specificity may be called 'short-is-good'. A widely accepted criteria for specificity of an antisense oligo is how well it can distinguish between its targeted sequence and a nontargeted sequence differing by only one base. In general, an oligo's ability to discriminate on the basis of a single base mismatch increases as its length decreases, reaching maximum discrimination at a length corresponding to, or just slightly greater than the MIL value for that structural type. Thus, a PNA of about 10 or 11 subunits in length would be expected to have maximum single-base mis-pairing discrimination for that structural type, while a Morpholino of about 15 or 16 subunits in length would be expected

to exhibit maximal single-base mis-pairing discrimination for that structural type. Since a single base mis-pairing in a 10-mer PNA/RNA duplex (10% of bases mis-paired) has a substantially larger impact on duplex stability than a single base mis-pair in a 15-mer Morpholino/RNA duplex (7% of bases mispaired), the higher-affinity PNAs provide a substantial advantage over lower-affinity Morpholinos for applications requiring single-base mis-match discrimination. Such applications include targeting single nucleotide polymorphisms (SNPs) and targeting point mutations.

Another strategy is that 'longer-is-better'. While the challenge of discriminating between a single base difference has received much attention in the antisense field, and is well met by short, high-affinity oligos such as PNAs, for antisense applications in complex systems the principal challenge is quite different. Such mainstream applications include determining the function of newly-sequenced genes; generating morphants<sup>9,27</sup> in embryos; validating targets in drug development programs; and developing therapeutics for viral diseases and cancers. The challenge in these complex systems is to strongly inhibit a targeted RNA (achieve high efficacy) without inadvertent inhibition of any other RNAs in the system (i.e., to achieve high specificity). Stated differently, what is needed in complex systems is an oligo which affords high efficacy, while also rigorously discriminating between its target RNA and thousands to tens of thousands of non-target RNAs.

To appreciate the challenge of obtaining both high efficacy and high specificity in a complex system such as a human, first consider the extreme case of a hypothetical ultra-high affinity 5-mer modifier-type oligo that has an MIL (Minimum Inactivating Length) of 5. Most RNAs in the pool of RNA transcripts will contain an average of about 4 copies of any given 5-mer sequence, based on an average RNA transcript length of about 4000 bases. Thus, if all sequences in the RNA transcripts were targetable, then one would expect this 5-mer oligo not only to inactivate its desired targeted RNA species, but also to inactivate nearly all other RNA species in the system.

For the case of a blocker-type oligo (such as a PNA or Morpholino), the specificity situation is not quite as bleak. This is because blocker-type oligos are generally only targetable against about 5% of the bases in a typical RNA transcript (cf. Figure 2a). As a consequence, an ultra-high affinity 5-mer blocker oligo is only expected to inadvertently inhibit about 20% of the non-targeted RNAs in the system – but this is still far

too little specificity for most applications in complex systems.

This brings us to what I believe are two crucial design requirements for an antisense oligo suitable for achieving both high efficacy and high specificity in a complex system. First, the oligo's MIL (Minimum Inactivating Length) value must be sufficiently large that the oligo has little chance of inadvertent inactivation of non-targeted species in the system's entire pool of RNA transcripts. Second, in order to achieve high efficacy the oligo's length should be appreciably longer than its MIL.

In regard to a lower limit for the MIL of oligos suitable for use in a complex *in vivo* system, for human use the current estimates are that the pool of RNA transcripts (before splicing) comprise about 30,000 species<sup>29</sup>. If pre-spliced RNA transcripts average on the order of about 4,000 bases in length, this gives approximately 120 million unique-sequence bases in the RNA pool, of which an estimated 5% (about 6 million bases) are targetable by advanced blocker type oligos (see Figure 2a). Table 4 below gives estimated numbers of RNA species in a human which would be inadvertently inhibited by oligos having the indicated MIL values and lengths corresponding to those values.

The values in Table 4 suggest that in order to have a reasonable chance of no inadvertent targets in a complex system comprising about 6 million bases of targetable unique-sequence RNA, an oligo should have an MIL value of about 12 or greater.

A 12-mer oligo with an MIL of 12 should have excellent specificity in a system as complex as a human; nonetheless, that 12-mer also would have only marginal efficacy. Since increasing binding affinity in order to increase efficacy would decrease the MIL below the value needed for high specificity in this complex system, the remaining option is to increase efficacy by increasing the oligo's length. As for the suitable length for achieving high efficacy, we generally find that Morpholino oligos 25 bases in length can provide high efficacies (90% to 100% target inhibition) at modest concentrations (100 nM to 1000 nM) in cell-free test systems where actual oligo concentration is known. 3,4,30,31 However, increasing the length of the oligo in order to increase its efficacy also leads to a modest increase in the number of potential inadvertent targets for that oligo. To illustrate, if the MIL for an oligo of a given structural type is about 15 (the case for Morpholinos) and the oligo length is 25, that oligo actually contains 11 different 15-mer sequences, each with its own potential for inadvertent

Table 5. Estimated numbers of inadvertent targets in RNA pool of 6 million targetable bases

MIL value	Estimated number of inadvertent targets for 25-mer oligos
8	1650
9	390
10	92
11	21
12	5.7
13	1.5
14	0.4
15	0.1

inhibition of non-targeted sequences. Thus, when the greater length needed for high efficacy is factored in, estimated numbers of inadvertent targets in the human RNA pool as a function of MIL are calculated as:

### Inadvertent targets =

(pool complexity /  $4^{MIL}$ ) (oligo length – MIL + 1)

Using this equation, estimated numbers of inadvertent targets in the human RNA pool are tabulated for 25-mer oligos as a function of the oligos' MIL values

The values in Table 5 suggest that to achieve high specificity in a human, a high-efficacy 25-mer should have an MIL of about 14 or greater. Thus, I predict that to achieve both high efficacy and high specificity in a human one should use an oligo structural type with an MIL value of about 14 or greater, and the oligo length should be on the order of 50% to 100% longer than the MIL value. Not unexpectedly, the lower-affinity Morpholinos (MIL  $\sim$ 15) fit these design criteria appreciably better than the higher-affinity PNAs (MIL about 10).

To test this theoretical prediction concerning how to achieve both high efficacy and high specificity in a complex system, we have carried out experiments to assess relative specificity of S-DNA, PNA, and Morpholino oligos in a test designed to emulate a high-complexity system.<sup>4,28</sup> In these experiments, two oligos of each structural type were used. One oligo of each type was perfectly complementary to its targeted mRNA (globin leader sequence) to provide a measure of total inhibition achieved by that structural type as a function of oligo concentration. The other oligo

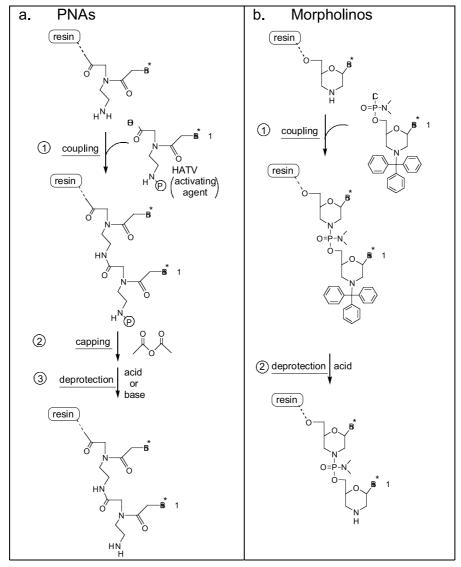


Figure 4. Representative oligo assembly cycles.

 $\textit{Table 4.} \ \ \text{Estimated numbers of inadvertent targets in RNA pool of 6 million targetable bases}$ 

MIL value (X)	Sequence permutations (4 <sup>x</sup> )	Estimated number of inadvertent targets $(6,000,000 \ / \ 4^x)$
8	65,500	92
9	262,000	23
10	1,050,000	6
11	4,190,000	1.4
12	16,800,000	0.4
13	67,100,000	0.09
14	268,000,000	0.02
15	1,070,000,000	0.006

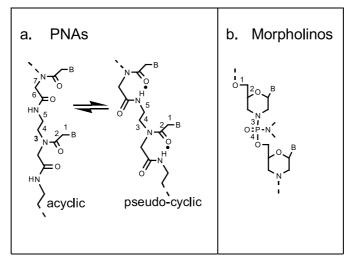


Figure 5. Backbone bonds with relatively free rotation.

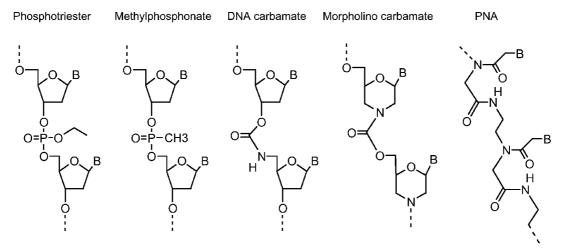


Figure 6. Non-ionic oligos with low aqueous solubilites.

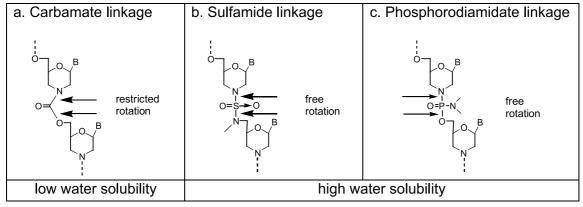


Figure 7. Morpholino oligos with varying aqueous solubilities.

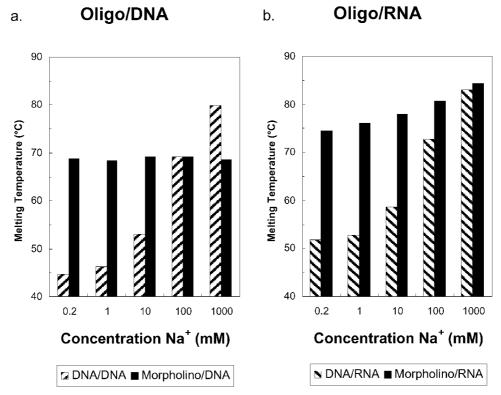


Figure 8. Salt dependence of Tm values for oligo/DNA and oligo/RNA duplexes.

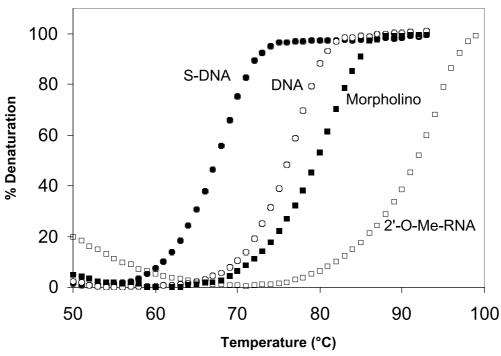
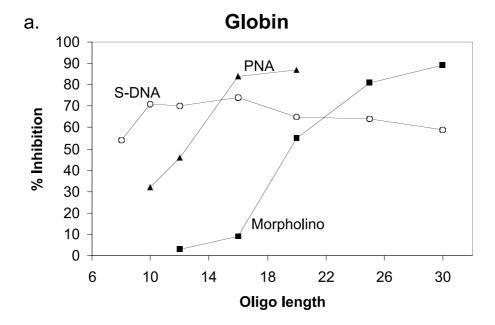


Figure 9. Thermal transitions of 20-mer oligo/RNA duplexes.



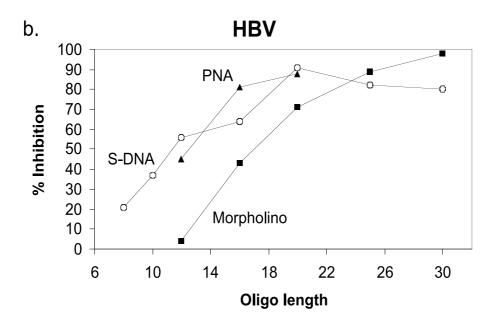


Figure 10. Antisense activity as a function of oligo length.

of that type incorporated mis-pairing to that same target sequence, with the longest run of perfect pairing comprising 10 contiguous base-pairs, to provide a reasonable emulation of the estimated level of sequence homology likely to be encountered in the RNA pool within a representative human cell. For the S-DNA and Morpholino structural types 25-mer oligos

were used and the mis-paired oligos contained four mis-pairs to the target sequence. For the PNA structural type 20-mer oligos were used and the mis-paired oligo contained three mis-pairs.

Specific inhibition was calculated as the difference between the inhibition value for the perfectly-paired oligo and the inhibition value for the mis-paired oligo

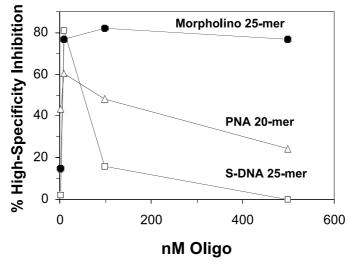


Figure 11. High-specificity component of inhibition by three structural types.

at each concentration. This provides a measure of the high-specificity inhibition achieved by that structural type as a function of concentration. Figure 11 shows a plot of this high-specificity component.

In accord with the length-versus-activity results in Figure 10 and the calculated data in Table 5, the experimental results in Figure 11 demonstrate that in this test emulating a high-complexity system, the high-MIL Morpholinos (MIL ~15) indeed exhibit substantially better sequence specificity over a wider concentration range than the lower-MIL PNAs (MIL about 10), which in turn exhibit substantially better sequence specificity over a wider concentration range than S-DNAs (MIL ~8). These results provide support for Morpholinos being a preferred structural type for applications in complex systems.

### Delivery into cultured cells

Until the mid-1980s most antisense experiments were carried out in cell-free test systems where the focus was on assessing prospective structural types for directly inhibiting translation of their targeted mRNAs. By the late 1980s and early 1990s, however, the antisense field had evolved to a stage where experiments were being carried out with cultured cells, at which point serious problems were encountered. Studies by several groups elucidated one particular problem – in cultured cells, neither ionic nor non-ionic antisense oligos can diffuse across cell membranes at any reasonable rate. 32,33 Instead, much evidence suggests that antisense oligos enter cultured cells via endocytosis and subsequently most or all of the oligos are

degraded, remain trapped in the endosome/lysosome compartment, or are exocytosed back to the extracellular medium.<sup>34</sup> Thus, under normal conditions antisense oligos fail to attain entry into the cytosol/nuclear compartment where their targeted sequences reside.

This delivery challenge has led to wide-ranging efforts to develop effective methods for delivering antisense oligos into the proper subcellular compartment of cultured cells. To a large extent these efforts have been successful, though toxicity from the delivery reagents remains a significant limitation for most of the delivery methods. Of particular note, in the past few years a wide range of components have been investigated for delivering PNAs into the cytosol/nuclear compartment of cells<sup>35–42</sup> and some of these may also hold promise for *in vivo* delivery.

In our own experience, delivery methods which work with Morpholinos generally also work well with PNAs. For example, scrape delivery is one method that is simple, effective and reliable for delivering both PNAs and Morpholinos into adherent cells. 43,44 It is also one of the few methods which work in the presence of high concentrations of serum.

A new and even more effective method suitable for delivering both Morpholino and PNA oligos entails complexing the non-ionic oligo with a partially complementary DNA oligo and then mixing this partial duplex with the weakly-basic polyamine, ethoxylated polyethyleneimine (EPEI), after which the composite complex is added to cells. The EPEI, which is only partially ionized at pH 7, serves both to bind electrostatically to the negatively-charged DNA component

229

of the oligo/DNA duplex, and to bind electrostatically to negatively-charged cell surfaces, effecting rapid endocytosis of the oligo/DNA/EPEI complex. It is believed that when the pH drops within the endosome, the EPEI is further ionized to the point where its charge density is sufficient to permeabilize the endosomal membrane, allowing release of the oligo into the cytosol of the cell. This 'Special Delivery' method (see also: www.gene-tools.com) is effective with a broad range of cell types, is quite efficient with both adherent and non-adherent cell types, and is less damaging to cells compared to most other delivery methods. Regrettably, the method does not work well in the presence of high concentrations of serum.

### Delivery in vivo?

While methods for delivering antisense oligos to the cytosol/nuclear compartment of cultured cells are now fairly well developed and reliable, most or all of those methods appear to be ineffective and/or too toxic for use in vivo. In light of these limitations in applying successful cultured-cell delivery methods in vivo, it came as a considerable surprise to many in the antisense field when reports began to circulate in the mid-1990s that by some as-yet-undefined mechanism, antisense oligos are able to gain entry into the cytosol/nuclear compartment of cells in vivo, 46,47 particularly in the liver and kidney. Subsequent to these reports, there have been additional reports implying successful in vivo delivery. In this context, it is widely touted that one S-DNA oligo (Vitravene) has been approved by the Food and Drug Administration of the USA for *in vivo* therapeutic application in humans, <sup>48</sup> and this is commonly construed to be definitive proof that antisense oligos (or at least S-DNAs) readily gain entry into the cytosol/nuclear compartment of cells in vivo. However, this 'proof' of effective cytosol/nuclear delivery in vivo is less impressive than it may appear, particularly in light of evidence that this particular S-DNA (ISIS 2922) does not function by an antisense mechanism within cells, but instead probably functions largely or solely in the extracellular milieu as an immune stimulatory agent due to CpG-containing sequences at each end of the oligo.<sup>49</sup>

Further evidence for effective cytosol/nuclear delivery *in vivo* has come from reports from AVI Bio-Pharma. This company developed a Morpholino oligo (Resten NG) which was reported to be effective in inhibiting restenosis following balloon angioplasty. <sup>50,51</sup> However, it should be appreciated that in this particu-

lar application, connections between cells in the artery wall are seriously perturbed during the course of the angioplasty procedure, and such perturbations have been reported to permeabilize plasma membranes of cells in the artery wall for a significant period of time following the scraping procedure.<sup>52</sup> It is believed that such cell permeabilization in the artery wall probably occurs by a mechanism similar to that which allows rapid cytosolic entry of antisense oligos during scrape delivery of cultured cells.<sup>43</sup> The key point here is that while effective delivery into the proper subcellular compartment may be achieved in AVI BioPharma's particular in vivo application, nonetheless, this does not imply that effective cytosol/nuclear delivery will also occur equally well in other in vivo applications where the cells are not mechanically perturbed.

Still another increasingly popular antisense application which provides evidence for effective cytosol/nuclear delivery in vivo is the successful use of Morpholinos for generating morphants (i.e., antisensemediated morphological changes which mimic mutational changes) in early-stage embryos of sea urchins, frogs, and zebra fish. 9,27 Again, appearances may be deceiving in regard to delivery, at least in the case of zebrafish. This is because the antisense oligos are injected into the zebrafish eggs before or very shortly after fertilization, at which time the normal permeability barriers between embryonic cells have not yet formed. Only somewhat after the 32-cell stage in zebrafish embryos do the normal permeability barriers begin to form between cells of the developing embryo. 53,54 Thus, when antisense oligos are injected into such eggs or embryos just beginning to undergo cell divisions, those antisense oligos have full access to all cells of the organism, and will reside and function within all those cells through multiple cell divisions. 16 Preliminary results from collaborators at the University of Oregon suggest that when antisense oligos are instead injected into the yolk or the vascular compartment of later stage embryos wherein the normal cellular permeability barriers have formed, those oligos fail to generate the expected phenotypic changes, suggesting a lack of reliable in vivo delivery.

Further evidence suggesting that Morpholino oligos do not readily cross cell membranes *in vivo* comes from studies with frog eggs/embryos, whose cells exhibit normal permeability barriers immediately after the first cell division. In this system, when fluorescent-tagged Morpholinos are injected into one cell of a 2-cell stage frog blastomere, the oligos remain only in the direct descendents of that injected cell (one side

of the developing embryo), at least through the freeswimming tadpole state (stage 43). The same basic result was also found in a functional test wherein the Morpholino was targeted against a stably-integrated transgene expressing green fluorescent protein.<sup>16</sup>

Thus, while many scientists in the antisense field contend that antisense oligos readily enter the proper subcellular compartment of many or all cells *in vivo*, many remain skeptical of most claims for antisense activity *in vivo*, at least in those cases where no delivery mechanism is apparent and no delivery component was used to achieve entry into the cytosol/nuclear compartment of the cells.

There are a number of important reasons for continued skepticism concerning *in vivo* delivery of bare antisense oligos:

- 1. One would expect that cells *in vivo* should not be significantly more permeable than cells in culture to large polar molecules such as antisense oligos, and this expectation appears to be supported by multiple studies with vertebrate embryos. For instance, preliminary studies indicate that a Morpholino oligo which generates a distinctive phenotypic change (fluorescent blood) when injected into early-stage zebrafish embryos fails to generate that same phenotype when injected into later-stage embryos wherein normal permeability barriers have formed. Further, Morpholino oligos injected into one cell of a 2-cell stage frog blastomere remain on one side of the frog embryo through the free-swimming tadpole stage. <sup>16</sup>
- 2. Early attempts to use 'naked' antisense oligos to inhibit virus infections in mice and other model organisms, where a definitive answer would be expected if the oligos were effective and high levels of *in vivo* delivery occurred, appear to have been relatively ineffective.
- 3. In most reports of *in vivo* antisense activity, S-DNAs were utilized, which are well known to generate multiple non-antisense effects due to their actions in the extracellular medium and at cell surfaces.<sup>55,56</sup> It is noteworthy that several of those non-antisense effects have been shown to be sequence dependent which can lead to erroneous conclusions even when apparently valid control oligos are used in the experiments.
- 4. Experiments targeting cellular genes in whole animals inherently entail many more uncertainties than targeting defined marker genes transfected into cultured cells, and results *in vivo* are often indirect and more open to interpretation. These greater uncertainties and less direct results in animal studies raise the chances that positive results are in reality just normal

statistical fluctuations, flaws in experimental design, or any of a host of other problems common to complex experimental systems.

5. It appears unlikely that all of the major pharmaceutical companies, who together invested billions of dollars in antisense research in the late 1980s and early 1990s, would have abandoned (in the late 1990s) their attempts to develop antisense therapeutics if they had been able to repeat the claimed *in vivo* successes of the small biotech antisense companies.

Notwithstanding this somewhat pessimistic assessment, I would like to conclude this section with two positive statements regarding *in vivo* delivery:

First, I believe that achieving safe and effective delivery of antisense oligos into the cytosol/nuclear compartment of a wide variety of cell types *in vivo* probably constitutes the last major challenge which must be met in order for antisense oligos to fulfill their great promise of safe and effective therapeutics for a broad range of viral diseases, cancers, possibly autoimmune diseases, and a host of other currently intractable disease states.

Second, based on recent advances by several research groups including GeneTools, it appears likely that this delivery challenge will be met soon, perhaps within the coming five years.

### Applications

### Research tools

PNAs have been used for an unusually wide variety of novel research applications. Probably because of their exceptionally high binding affinity and unusually flexible backbones, PNAs have proven particularly useful for forming triplexes with DNA and RNA. With duplex DNA targets, in low salt conditions (to destabilize the DNA/DNA duplex) an all-pyrimidine PNA can invade a targeted purine or pyrimidine stretch of duplex DNA to form a PNA/DNA/PNA triplex and a single-stranded displacement loop comprising the pyrimidine-containing strand of DNA.<sup>17</sup> For the case of single-stranded, all-purine RNA targets, PNAs also can form ultra-stable PNA/RNA/PNA triplexes which have the unique capability of blocking translocation of ribosomes - even in downstream amino acid-coding regions of a targeted mRNA. Thus, for these rare target sequences, such specially-designed PNAs can be effective in a region of the mRNA which is normally only targetable by modifier-type antisense structural types.6

Because of their exceptionally high binding affinity, PNAs also excel in targeting inherently short RNA sequences, such as the short RNA sequence exposed in the telomerase enzyme. 57,58

A particularly promising exploitation of high affinity PNAs is their use as antisense antibacterials, where the limited porosity of bacterial cell walls largely preclude use of antisense oligos longer than about 12 to 14 bases. A major advance in this application was recently reported wherein addition of a short cell wall-permeabilizing peptide to a 12-mer PNA dramatically increased the PNAs antibacterial efficacy. <sup>59</sup>

As noted in earlier sections, PNAs are also attractive because of their compatibility with peptide synthesis conditions. This allows one to synthesize a PNA, and then while the PNA is still protected and on its synthesis resin, amino acids are added stepwise to form peptide adducts suitable for enhancing delivery into cells<sup>35,60</sup> or for other applications such as diagnostics.

PNAs have been used in a novel extra-cellular therapeutics application wherein an antibody/PNA adduct is used to bind to cancer-specific antigens on the surface of cancer cells *in vivo*. This is followed by addition of a second complementary PNA to which is attached a suitable radioisotope. The isotope-carrying PNA then rapidly pairs to its complementary PNA linked to the antibody bound to the cancer cells. This process effectively concentrates the isotope in proximity to the cancer cells – thereby substantially increasing the damage to the cancer cells and decreasing damage to the non-cancer cells. <sup>61</sup>

PNAs also have been investigated for use in a variety of experimental systems for detecting and quantitating human or animal genetic sequences.  $^{62-66}$ 

In contrast to the wide-ranging applications of PNAs, so far Morpholinos have been used primarily for classical antisense applications in complex systems. Such applications include correcting splicing errors in pre-mRNAs in cultured cells<sup>44,45</sup> and in extra-corporal treatment of cells from thallasemic patients.<sup>67</sup>

Morpholinos are often used for classic antisense inhibition of targeted mRNAs in cultured cells when both high efficacy and high specificity are desired. 68–70 In this context, a Morpholino targeted against the C-myc mRNA is in Phase 3 clinical trials for prevention of restenosis following balloon angioplasty. 50 In this application it is likely that delivery is achieved *in vivo* via scrape delivery into cells of the artery wall during the angioplasty procedure.

Perhaps the most demanding application of Morpholinos is in developmental biology. 9,27,71-74 For this application it is necessary to inject a very small volume of a high concentration of oligo into eggs or early-stage embryos<sup>9</sup> or to electroporate oligos into specific tissues in later-stage embryos.<sup>74</sup> Obviously, oligos for this application must be very soluble in water (i.e., multi-millimolar). Such oligos also must exhibit extremely high sequence specificity and have negligible toxicity and negligible non-antisense effects. The need for exquisite specificity is because within a brief period of time (a few days) the rapidly developing organism expresses most of its entire set of genes and so the antisense oligo must efficiently inhibit its targeted mRNA without significantly affecting any of the very large number of other mRNA species which are present at one time or another during embryogenesis (but mostly absent from terminally differentiated cells). Developmental biology applications also require that the oligos be stable in biological systems for long periods of time. To the best of our knowledge, to date Morpholinos are the only antisense type shown to work predictably, specifically and without toxicity in this very demanding application.

A particularly interesting application in developmental biology is the use of Morpholinos to selectively target zygotic RNAs without concomitant inhibition of maternal RNAs coded by the same gene. This is achieved by targeting intron/exon splice junctions, which are present in the newly transcribed zygotic premRNAs, but absent from the already-spliced maternal mRNAs.

Another valuable strategy which can be utilized in studies with frog eggs is to inject the test Morpholino oligo into only one cell of a 2-cell stage blastomere. The other cell of the blastomere is either not injected or is injected with a control Morpholino oligo. In the course of development, all cells on one side of the embryo come from the injected cell and all cells from the other side of the embryo come from the non-injected or control-injected cell. It has been shown that in this manner one side of the embryo serves as an excellent control for the other side because Morpholino oligos in cells of one side of the embryo cannot pass over to cells of the other side of the embryo.<sup>16</sup>

### Clinical diagnostics

For the past few decades, it has appeared to many scientists involved in nucleic acid research that nucleic acid probes should be greatly superior to antibodies for detecting infectious diseases in the clinic. This is because nucleic acid probes (or other probes) can have higher binding affinities than antibodies as well as greater specificity than even monoclonal antibodies. Most importantly, generating a probe specific for a selected genetic sequence (analyte strand) is much simpler, faster, and more reliable than generating a corresponding pathogen-specific antibody. In spite of these apparent advantages, after several decades of development, probe diagnostics have only made minor inroads into the clinical diagnostics arena, with antibody-based diagnostics still enjoying nearly complete dominance.

There are three principal challenges which may be responsible for classic probe diagnostics having failed to gain a significant foothold in the clinic: 1. Stringency. Probes generally require precise control over stringency (i.e., salt, denaturant, temperature) during probe/target pairing, giving false positives if stringency is too low and false negatives if stringency is too high. This raises the specter of poor reliability or reproducability, which can be catastrophic in the context of clinical diagnostics.

- 2. Speed. For samples containing low concentrations of analyte strands, for example  $\sim 600$  virus particles per ml of blood, which corresponds to about 1 attomolar, pairing of probe to its complementary analyte strand typically requires many hours, or a relatively complex time-consuming, expensive, errorprone pre-amplification of a key portion of the analyte strand by polymerase chain reaction (PCR) or some analogous target amplification procedure.
- 3. Sensitivity. The concentration of analyte strands (e.g., viral genetic sequences) in clinical samples are often in the zeptomolar  $(10^{-21})$  or attomolar  $(10^{-18})$  range and probe diagnostic methods for detecting these very low analyte concentrations generally require complicated time-consuming and expensive pre-amplification of a portion of the analyte strand (such as by PCR) or use of complicated and expensive detection equipment not appropriate for a clinical setting.

One major exception to the limited sensitivity of probe diagnostics systems is the Branched DNA system developed at Chiron and now widely used in the clinic for quantitation of HIV.<sup>76</sup> Still, even the Branched DNA system is slow, complicated, and expensive compared to most antibody-based diagnostics.

High-affinity non-ionic probes, such as PNAs and Morpholinos, are well-suited to overcoming these many challenges currently impeding the use of probe diagnostics in most clinical applications.

With respect to stringency, the reason precise control of stringency is important with standard nucleic acid probes is that under low stringency conditions (i.e., too much salt or too little denaturant or too low of a temperature) the target sequence of the analyte strand, complementary to the probe, is largely unavailable for pairing to the probe because of extensive secondary structure in the analyte strand. Conversely, when the stringency is too high (i.e., too little salt or too much denaturant or too high of a temperature) the probe is unable to stably bind to its target sequence in the analyte strand.

In contrast to the case for standard ionic probes (e.g., DNA and RNA), when using a non-ionic probe and pairing in salt-free water, essentially all secondary structure in the analyte strand is disrupted due to electrostatic repulsion between backbones, allowing full access of the probe to its target sequence in the analyte strand. Further, because the probe has no backbone charge, these same salt-free conditions have little or no impact on the probe/target pairing (see Figure 8 and Reference 1). Thus, by using a non-ionic probe (PNA or Morpholino) the challenge of precisely controlling stringency can be disposed of simply by carrying out the pairing step in salt-free water at a temperature anywhere in a fairly large range (ambient to about 50 °C). As a consequence, use of non-ionic probes can significantly simplify the diagnostic system. More important, it can also greatly reduce the chance of false negatives and false positives.

With respect to speed, a method has been developed which allows very rapid pairing (seconds to a few minutes) between a probe and its target sequence in an analyte strand, even when that analyte strand is present at extremely low concentrations (zeptomolar) in a biological sample.<sup>77</sup> In one embodiment of that method a surface (e.g., microbeads or a porous frit) is derivatized with both a weakly-basic oligoamine (effective pKa  $\sim$ 6) and a non-ionic probe. When a biological sample buffered at about pH 5 (to assure ionization of the oligoamine) is contacted with that oligoamine/probe surface, all polyanionic nucleic acids, including any analyte strands, are adsorbed in seconds to that oligoamine/probe surface via electrostatic bonds between the cationic oligoamines and the anionic nucleic acids. The surface is then washed with salt-free water to disrupt secondary structures in the analyte strand and allow pairing between surfacebound probes and analyte strands held in immediate proximity to the probe due to electrostatic bonding to the interspersed oligoamines. This probe/target pairing is generally complete within a couple of minutes, even when the analyte strand was originally present at extremely low concentrations. The surface is next washed with pH 8 buffer to deionize the weakly basic oligoamines and thereby terminate the electrostatic bonding between the surface-bound oligoamines and nucleic acids. Washing the surface with pH 8 buffer effects removal of all nucleic acids except analyte strands still linked to the surface via Watson/Crick bonds between the surface-bound probes and target sequences of the analyte strands.

To summarize this rapid-pairing strategy, biological sample buffered at pH 5 is contacted with the oligoamine/probe surface to capture all nucleic acids. The surface is then washed with water to allow pairing between the probe and target sequences within any analyte strands. Several minutes later the surface is washed with pH 8 buffer to remove all non-analyte strands. By this means, pairing which would normally take many hours is achieved near quantitatively in several minutes.

In regard to sensitivity, in the research laboratory low-copy-number analyte strands are routinely detected either by selectively pre-amplifying a portion of the analyte strand (such as by PCR) or by highly amplifying a signal associated with the probe – where said amplification is generally complicated, labor intensive, and expensive. While these complexities and costs may be acceptable in a research setting, I believe they constitute a major impediment to use of probe diagnostics in most clinical applications.

Thus, it appears that the last major challenge in developing probe diagnostics suitable for the clinic is the development of a direct detection scheme which is capable of reliably detecting as few as about ten to a hundred analyte strands in a 1-5 ml biological sample, and which is also fast, simple, and cheap. While such a simple high-sensitivity direct detection capability appears not to be available at this time, GENE TOOLS is embarking on development of a direct detection method using novel structures and a unique scheme which exploits the special properties of non-ionic probes. This detection scheme actually comprises two key aspects, as follows: 1) a component effective to provide a very large signal (tens of thousands of fluorophores) per analyte strand; and, 2) a mechanism for dramatically reducing the typical level of background signal.

In light of past progress and expected upcoming developments, it is possible that probe diagnostics will finally win a major share of the clinical diagnostics market in the foreseeable future. I further believe that because of the compelling advantages afforded by non-ionic probes, the probe components in those clinical diagnostics will almost surely have non-ionic backbones.

### **Therapeutics**

It should be appreciated that both PNAs and Morpholinos are relatively new structural types, with the currently preferred embodiments only about a decade old. Furthermore, the rate of their development was substantially slowed during much of the past decade because until recently most of the economic resources available in the antisense field from government, the pharmaceutical industry, and investors have been focused on the seriously flawed S-DNAs.

In spite of the above factors, *in vivo* studies with bare PNAs and bare Morpholinos (i.e., oligos with no added delivery component) are now being undertaken by a number of groups – and results from at least some of these studies suggest that I might be mistaken in my belief that a delivery component will generally be required for effective delivery of antisense oligos into the cytosol/nuclear compartment of cells of living animals.

One study providing support for effectiveness of bare PNAs *in vivo* utilized a PNA targeted against the neurotensin receptor 1 and another PNA targeted against the opioid mu receptor. These bare PNAs were injected into the periaqueductal gray region in brains of rats. Each PNA produced the physiological response expected if it had down regulated its targeted mRNA in neuronal cells, and subsequent biochemical assessment of the brains of the treated rats indicated that the concentrations of the protein products coded by the targeted mRNAs were significantly reduced, as would be expected from an antisense mechanism.<sup>78</sup>

In regard to *in vivo* use of bare Morpholinos, AVI BioPharma, owner of the patents covering Morpholinos, is aggressively pursuing the development of a number of different Morpholino therapeutics. To date, none of these prospective Morpholino therapeutics contain a delivery component. In spite of this, AVI BioPharma and their multiple collaborating groups have reported positive results from studies in a variety of different animal species and in humans for Mor-

pholinos targeted against the mRNAs of a number of different genes. <sup>79–82</sup>

If the foregoing results are valid, and not just special cases of exceptional cell permeabilities in such sites as the brain and liver, then in light of the impressive properties of these advanced antisense structural types, it seems possible that in the future safe and effective antisense therapeutics (Morpholinos and/or PNAs) will be introduced for the treatment of a wide range of diseases.

Conversely, if the *in vivo* results with bare PNAs and bare Morpholinos prove to be invalid or merely special cases, then the introduction of a broad range of antisense therapeutics will likely be delayed until after safe and effective delivery components are developed.

Editor's note: As the inventor and commercial producer of Morpholinos, Dr. Summerton has disclosed his financial interest in Morpholino technology. Also, Dr. Summerton wishes to state that his first-hand experience with PNAs has been limited to experimental comparisons between Morpholinos, PNAs, 2'O-methyl RNAs, and S-DNAs with respect to properties expected to be important for diagnostic applications and for therapeutic applications against viral diseases and cancers.

### References

- Liam, G. and Nielsen, P.E. Progress in developing PNA as a gene-targeted drug. Antisense Nucl. Acid Drug Dev., 7 (1997) 431–437.
- Nielsen, P. and Egholm, M. (Eds.) Peptide Nucleic Acids. Norfolk: Horizon Scientific Press (1999).
- Summerton, J. and Weller, D. Morpholino antisense oligomers: design, preparation and properties. Antisense Nucleic Acid Drug Dev. 7 (1997) 187–195.
- Summerton, J. Morpholino antisense oligomers: the case for an RNase H-independent structural type. Biochim. Biophys. Acta, 1489 (1999) 141–158.
- Summerton, J. (1990) Polynucleotide assay reagent and method. Canadian Patent; 1, 268, 404. Summerton J, Weller D. (1993). Uncharged Morpholino-based polymers having phosphorous containing chiral intersubunit linkages. US Patent; 5, 185, 444.
- 6. Website: www.gene-tools.com
- Knudsen, H. and Nielsen, P.E. Antisense properties of duplex and triplex forming PNA. Nucl. Acids Res., 24 (1996) 494– 500
- Milner, N., Mir, K. and Southern, E. Selecting effective antisense reagents on combinatorial oligonucleotide arrays. Nat. Biotechnol., 15 (1997) 537–541.
- Ho, S., Bao, Y., Lesher, T., Malhotra, R., Ma, L., Fluharty, S. and Sakai R. Mapping of RNA accessible sites for antisense experiments with oligonucleotide libraries. Nat. Biotechnol. 16 (1998) 59.

- Ekker, S. Morphants: a new systematic vertebrate functional genomics approach. Yeast, 17 (2000) 302–306.
- Koch, T. PNA oligomer synthesis by BOC chemistry. In:
   P. Neilsen and M. Egholm (Eds.), Peptide Nucleic Acids. Norfolk: Horizon Scientific Press (1999) 21–37.
- Casale, R., Jensen, I.S. and Egholm, M. Synthesis of PNA oligomers by Fmoc Chemistry. In: P. Neilsen and M. Egholm (Eds.), Peptide Nucleic Acids. Norfolk: Horizon Scientific Press (1999) 39–50.
- Uhlmann, E., Greinerm B. and Breipohl, G. PNA/DNA chimeras. In: P. Neilsen and M. Egholm (Eds.), Peptide Nucleic Acids. Norfolk: Horizon Scientific Press (1999) 51–70.
- Stein, C.A. and Cohen, J.S. Phosphorothioate oligodeoxynucleotide analogues. In: J. Cohen (Ed.), Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression. Boca Raton: CRC Press (1989) 97–117.
- Demidov, V., Potaman, V.N., Frank-Kamenetskii, M.D., Buchardt, O., Egholm, M. and Nielsen, P.E. Stability of peptide nucleic acids in human serum and cellular extracts. Biochem. Pharmacol., 48 (1994) 1309–1313.
- Hudziak, R.M., Barofsky, E., Barofsky, D.F., Weller, D.L., Huang, S.B. and Weller, D.D. Resistance of Morpholino phosphorodiamidate oligomers to enzymatic degradation. Antisense Nucl. Acid Drug Dev., 6 (1996) 267–272.
- Nutt, S.L., Bronchain, O.J., Hartley, K.O. and Amaya, E. Comparison of Morpholino based translational inhibition during the development of *Xenopus laevis* and *Xenopus tropicalis*. Genesis, 30 (2001) 110–113.
- Larsen, H.J. and Nielsen, P.E. Characterization of PNAdsDNA strand displacement complexes. In: P. Neilsen and M. Egholm (Eds.), Peptide Nucleic Acids. Norfolk: Horizon Scientific Press (1999) 221–240.
- Hanvey, J.C., Peffer, N.J., Bisi, J.E., Thomson, S.A., Cadilla, R., Josey, J.A., Ricca, D.J., Hassman, C.F., Bonham, M.A. and Au, K.G. *et al.* Antisense and antigene properties of peptide nucleic acids. Science, 258 (1992) 1481–1485.
- Selecting sequences for Morpholinos, targeting guidelines: http://www.gene-tools.com/Ordering/Ordering1/ body ordering1.HTML
- Selecting sequences for PNAs, targeting guidelines: http://www.appliedbiosystems.com/support/seqguide.cfm
- Miller, P. Non-ionic antisense oligonucleotides: Oligonucleotide alkylphosphotriesters. In: J. Cohen (Ed.), Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression. Boca Raton: CRC Press (1989) 82–85.
- Miller, P. Non-ionic antisense oligonucleotides: Oligonucleotide methylphosphonates. In: J. Cohen (Ed.) Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression. Boca Raton: CRC Press (1989) 85–92.
- Stirchak, E., Summerton, J. and Weller, D. Uncharged stereoregular nucleic acid analogues. 1. Synthesis of a cytosinecontaining oligomer with carbamate internucleoside linkages. J. Org. Chem., 52 (1987) 4202–4206.
- Stirchak, E., Summerton, J. and Weller, D. Uncharged stereoregular nucleic acid analogues. 2. Morpholino nucleoside oligomers with carbamate internucleoside linkages. Nucl. Acids Res., 17 (1989) 6129–6141.
- Gildea, B.D., Casey, S., MacNeill, J., Perry-O'Keefe, H., Sørensen, D and Coull, J.M. Tetrah. Lett., 39 (1998) 7255.
- Kang, H., Chou, P., Johnson, C., Weller, D., Huang, S, and Summerton, J. Stacking interactions of ApA analogues with modified backbones. Biopolymers, 32 (1992) 1351–1363.
- 28. Genesis, 30 (2001) 3: Entire issue.

- Summerton, J., Stein, D., Huang, S., Matthews, P., Weller, D. and Partridge, M. Morpholino and Phosphorothioate antisense oligomers compared in cell-free and in-cell systems. Antis. Nucl. Acids Drug Dev., 7 (1997) 63–70.
- Venter, J.C. *et al.* The sequence of the human genome. Science, 291 (2001) 1304–1351.
- Stein, D., Foster, E., Huang, S.B., Weller, D. and Summerton, J. A specificity comparison of four antisense types: Morpholino, 2'-O-methyl RNA, DNA, and Phosphorothioate DNA. Antis. Nucl. Acids Drug Dev., 7 (1997) 151–157.
- Summerton, J., Stein, D., Huang, S.B., Matthews, P., Weller, D. and Partridge, M. Morpholino and Phosphorothioate antisense oligomers compared in cell-free and in-cell systems. Antis. Nucl. Acids Drug Dev., 7 (1997) 63–70.
- Akhtar, S., Basu, S., Wickstrom, E. and Juliano, R. Interaction of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). Nucl. Acids Res., 19 (1991) 5551–5559.
- Akhtar, S. and Juliano, R. Adsorption and efflux characteristics of modified oligodeoxynucleotides from liposomes. Proc. Am. Assoc. Cancer Res. 32 (1991) 333.
- Neckers, L. Cellular internalization of oligodeoxynucleotides. In: S. Crooke and B. Lebleu B. (Eds.), Antisense Research and Applications. Boca Raton: CRC Press (1993) 451–460.
- Simmons, C.G., Pitts, A.E., Mayfield, L.D., Shay, J.W. and Corey, D.R. Synthesis and membrane permeability of PNApeptide conjugates. Bioorg. Med. Chem. Lett. 7 (1997) 3001.
- Pooga, M., Soomets, U., Hällbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J.X., Xu, X.J., Weisenfeld-Hallin, Z., Hökfelt, T., Bartfai, T. and Langel, Ü. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. Nat. Biotechnol., 16 (1998) 857– 861.
- Tyler, B.M., McCormick, D.J., Hoshall, C.V., Douglas, C.L., Jansen, K, Lacy, B.W., Cusack, B. and Richelson, E. Specific gene blockade shows that peptide nucleic acids readily enter neuronal cells *in vivo*. FEBS Lett., 421 (1998) 280–284.
- Pardridge, W.M., Boado, R.J. and Kang, Y.S. Vector-mediated delivery of a polyamide ('peptide') nucleic acid analogue through the blood-brain barrier *in vivo*. Proc. Natl. Acad. Sci, 92 (1995) 5592–5596.
- Aldrain-Herrada, G., Desarménien, M.G., Orcel, H., Boissin-Agasse, L., Méry, J., Brugidou, J. and Rabie, A. A peptide nucleic acid (PNA) is more rapidly internalized in cultured neurons when coupled to a retro-inverso delivery peptide. The antisense activity depresses the target mRNA and protein in magnocellular oxytocin neurons. Nucl. Acids Res., 26 (1998) 4910–4916.
- Basu, S. and Wickstrom, E. Synthesis and characterization of a peptide nucleic acid conjugated to a D-peptide analog of insulin-like growth factor 1 for increased cellular uptake. Bioconj. Chem., 8 (1997) 481–488.
- Good, L. and Nielsen, P.E. Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA. Nat. Biotechnol., 16 (1998) 355–358.
- Faruqi, A.F., Egholm, M. and Glazer, P.M. Peptide nucleic acid-targeted mutagenesis of a chromosomal gene in mouse cells. Proc. Natl. Acad. Sci. USA, 95 (1998) 1398–1403.
- Partridge, M., Vincent, A., Matthews, P., Puma, J., Stein, D. and Summerton, J. A simple method for delivering Morpholino antisense oligos in to the cytoplasm of cells. Antis. Nucl. Acids Drug Dev., 6 (1996) 169–175.

- Morcos, P.A. Gene switching: analyzing a broad range of mutations using steric block antisense oligonucleotides. Meth. Enzymol., 313 (1999) 174–189.
- Morcos, P.A. Achieving efficient delivery of Morpholino oligos in cultured cells. Genesis, 30 (2001) 94–102.
- Monia, B., Johnston, J., Geiger, T., Muller, M. and Fabbro,
   D. Antitumor activity of a Phosphorothioate antisense oligodeoxynucleotide targeted against C-raf kinase. Nat. Med., 2 (1996) 668–675.
- 48. Monia, B., Sasmor, H., Johnston, J., Freier, S., Lesnik, E., Muller, M., Geiger, T., Altmann, K., Moser, H. and Fabbro, D. Sequence-specific antitumor activity of a Phosphorothioate oligodeoxyribonucleotide targeted to human C-raf kinase supports an antisense mechanism of action *in vivo*. Proc. Natl. Acad. Sci. USA, 93 (1996) 15481–15484.
- Baker, B.F. and Monia, B.P. Novel mechanisms for antisensemediated regulation of gene expression. Biochem. Biophys. Acta, 1489 (1999) 3–18.
- Quote from Stein, C. and Krieg, A., Non-antisense effects of oligodeoxynucleotides. In: C. Lichtenstein C. and Nellen, W. (Eds.) Antisense Technology. Oxford: IRL Press, 1997: 260. '... non-antisense effects of PS oligos are also pointed out by a close examination of the oligo ISIS 2922, an 'antisense' oligo with remarkably potent antiviral effects against cytomegalovirus (CMV). ISIS 2922 is reported to have encouraging in vivo efficacy in early clinical trials against CMV retinitis. The sequence of this oligo is quite interesting because it has two atypical CpG motifs: there is a GCG at both the extreme 5' and 3' ends (79). Although not fully recognized at the time, published studies on ISIS 2922 demonstrate that its antiviral effect cannot be due to 'antisense' since internal mismatch control oligos lost very little antiviral activity despite a severe drop in the Tm. On the other hand, deletion of a single base from one of the CpG motifs caused a 40% drop in antiviral efficacy, and deletion of a single base from both of the CpG motifs abolished antiviral effect despite little change in the Tm for hybridization to the supposed mRNA target (79)'.
- Kipshidze, N., Moses, J., Shankar, L.R. and Leon, M. Perspectives on antisense therapy for the prevention of restenosis. Curr. Opin. Mol. Ther., 3 (2001) 265–277.
- 52. Kipshidze, N., Keane, E., Stein, D., Chawla, P., Skrinska, V., Shandar, L.R., Komorowski, R., Haudenschild, C., Leon, M., Keelan, M.H., Moses, J. and Iversen, P. Local delivery of antisense phosphorodiamidate morpholino oligomer; Resten-NG inhibits myointimal hyperplasia following balloon angioplasty. In: F. Navarro-Lopez (Ed.), XXIst Congress of the European Society of Cardiology. Italy: Monduzzi Editore (1999) 463–467.
- Farrell, C., Bready, J., Saufman, S., Quian, Y. and Burgess, T. The uptake and distribution of Phosphorothioate oligonucleotides into vascular smooth muscle cells *in vitro* and in rabbit arteries. Antis. Res. Dev., 5 (1995) 175–183.
- Kimmel, C.B. and Law, R.D. Cell lineage of zebrafish blastomeres. I. Cleavage pattern and cytoplasmic bridges between cells. Dev. Biol., 108 (1985) 78–85.
- Kimmel, C.B. and Law, R.D. Cell lineage of zebrafish blastomeres. II. Formation of the yolk syncytial layer. Dev. Biol., 108 (1985) 86–93.
- Stein, C. and Krieg, A. Non-antisense effects of oligodeoxynucleotides. In: C. Lichtenstein and W. Nellen (Eds.) Antisense Technology. Oxford: IRL Press (1997) 241–264.
- Stein, C. Does antisense exist? Nat. Med., 1 (1995) 1119– 1121.

- Norton, J.C., Piatyszek, M.A., Wright, W.E., Shay, J.W. and Corey, D.R. Inhibition of human telomerase activity by peptide nucleic acids. Nat. Biotechnol., 14 (1996) 615–619.
- Hamilton, S.E., Pitts, A.E., Katipally, R.R., Jia, X., Rutter, J.P., Davies, B.A., Shay, J.W., Wright, W.E. and Corey, D.R. Identification of determinants for inhibitor binding within the RNA active site of human telomerase using PNA scanning. Biochemistry, 36 (1997) 11873–11880.
- Good, L., Awasthi, S.K., Dryselius, R., Larsson, O. and Nielson, P.E. Bactericidal antisense effects of peptide-PNA conjugates. Nat. Biotechnol., 19 (2001) 360–364.
- Tyler, B.M., McCormick, D.J., Hoshall, C.V., Douglas, C.L., Jansen, K., Lacy, B.W., Cusack, B. and Richelson, E. Specific gene blockade shows that peptide nucleic acids readily enter neuronal cells in vivo. FEBS Lett., 421 (1998) 280–284.
- Rusckowski, M., Qu, T., Chang, F. and Hnatowich, D.J. Pretargeting using peptide nucleic acid. Cancer, 80 (1997) (12 suppl) 2699–2705.
- 63. Thisted, M., Just, T., Pluzek, K.J., Hyldig-Nielsen, J.J., Nielsen, K.V., Mollerup, T.A., Stender, H., Rasmussen, O.F., Adelhorst, K. and Godtfredsen, S.E. Application of peptide nucleic acid probes for *in situ* hybridization. In: P. Nielsen and M. Egholm (Eds.) Peptide Nucleic Acids: Protocols and Applications. Wymondham: Horizon Scientific Press (1999) 99–118
- Matysiak, S., Würtz, S., Hauser, N.C., Gausepohl, H. and Hoheisel, J.D. PNA-arrays for nucleic acid detection. In: P. Nielsen and M. Egholm (Eds.), Peptide Nucleic Acids: Protocols and Applications. Wymondham: Horizon Scientific Press (1999) 119–128.
- Fiandaca, M.J., Hyldig-Nielsen, J.J. and Coull, J.M. PNA blocker probes enhance specificity in probe assays. In: P. Nielsen and M. Egholm (Eds.), Peptide Nucleic Acids: Protocols and Applications. Wymondham: Horizon Scientific Press (1999) 129–142.
- Griffin, T.J., Tang, W. and Smith, L.M. MALDI-TOF mass spectrometric detection of PNA hybrids for genetic analysis. In: P. Nielsen and M. Egholm (Eds.), Peptide Nucleic Acids: Protocols and Applications. Wymondham: Horizon Scientific Press (1999) 143–154.
- Wang, J. PNA biosensors for nucleic acid detection. In: P. Nielsen and M. Egholm (Eds.), Peptide Nucleic Acids: Protocols and Applications. Wymondham: Horizon Scientific Press (1999) 155–161.
- Lacerra, G., Sierakowska, H., Carestia, C., Fucharoen, S., Summerton, J., Weller, D. and Kole, R. Restoration of hemoglobin A synthesis in erythroid cells from peripheral blood of thalassemic patients. Proc. Natl. Acad. Sci. USA, 97 (2000) 9591–9596.
- Deng, J., Hua, K., Lesser, S.S. and Harp, J.B. Activation of signal transducer and activator of transcription-3 during proliferative phases of 3T3-L1 adipogenesis. Endocrinology, 141 (2000) 2370–2376.

- Lawn, R.M., Wade, D.P., Garvin, M.R., Wang, X., Schwartz, K., Porter, J.G., Seilhamer, J.J., Vaughan, A.M. and Oram, J.F. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. J. Clin. Invest., 104 (1999) R25–R31.
- Giles, R.V., Spiller, D.G., Clark, R.E. and Tidd, D.M. Antisense morpholino oligonucleotide analog induces missplicing of c-myc mRNA. Antis. Nucl. Acids Drug Dev., (Apr)9(2) (1999) 213–20.
- Heasman, J., Kofron, M. and Wylie, C. Beta-catenin signaling activity dissected in the early *Xenopus* embryo: a novel antisense approach. Devel. Biol., 222 (2000) 124–134.
- Nasevicius, A. and Ekker, S.C. Effective targeted gene knockdown in zebrafish. Nat. Genetics, 26 (2000) 216–220.
- Howard, E.W., Newman, L.A., Oleksyn, D.W., Angerer, R.C. and Angerer, L.M. SpKrl: a direct target of (beta)catenin regulation required for endoderm differentiation in sea urchin embryos. Development, 128 (2001) 365–375.
- Kos, R., Reedy, M.V., Johnson, R.L. and Erickson, C.A. The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. Development, 128 (2001) 1467–1479.
- Draper, B.W., Morcos, P.A. and Kimmel, C.B. Inhibition of zebrafish fgf8 pre-mRNA splicing with Morpholino oligos: a quantifiable method for gene knockdown. Genesis, 30 (2001) 154–156.
- Elbeik, T. et al. Quantitative and cost comparison of ultrasensitive human immunodeficiency virus type 1 RNA viral load assays. J. Clin. Micro., 39 (2000) 1113–1120
- Summerton, J.E., Weller, D.D. and Wages, J.M. Reagent and method for isolation and detection of selected nucleic acid sequences. US Patent; (2000), 6,060,246.
- Tyler, B.M., McCormick, D.J., Hoshall, C.V., Douglas, C.L., Jansen, K., Lacy, B.W., Cusack, B. and Richelson, E. Specific gene blockade shows that peptide nucleic acids readily enter neuronal cells in vivo. FEBS Lett. 421 (1998) 280–284.
- Arora, V. and Iversen, P. Redirection of drug metabolism using antisense technology. Curr. Opin. Mol. Ther., 3 (2001) 249–257
- Brand, R.M. and Iversen, P.L. Transdermal delivery of antisense compounds. Adv. Drug Develop. Rev., 44 (2000) 51–58.
- Aurora, V. and Iversen, P.L. Antisense oligonucleotides targeted to the p53 gene modulate rat liver regeneration. Drug. Metab. Disp., 28 (2000) 131–138.
- Qin, G., Taylor, M., Ning, Y.Y., Iversen, P. and Kobzik, L. *In vivo* evaluation of a morpholino antisense oligomer directed against TNF. Antis. Nucl. Acids Drug Develop., 10 (2000) 11–16.
- Stein, C.A. and Cohen, J.S. Phosphorothioate oligodeoxynucleotide analogues. In: J. Cohen (Ed.), Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression. Boca Raton: CRC Press (1989) 97–117.

# EXHIBIT 10

# Collins Dictionary

### HarperCollins Publishers

Westerhill Road Bishopbriggs Glasgow G64 2QT

Eleventh edition 2011

Reprint 10 9 8 7 6 5 4 3 2 1 0

© William Collins Sons & Co. Ltd 1979, 1986 © HarperCollins Publishers 1991, 1994 (Third updated edition), 1998, 2000, 2003, 2005, 2006, 2007, 2009, 2010, 2011

UK EDITION ISBN 978-0-00-743786-3

AUSTRALIAN EDITION ISBN 978-0-00-744523-3

NEW ZEALAND EDITION ISBN 978-0-00-744522-6

Collins® is a registered trademark of HarperCollins Publishers Limited

www.collinslanguage.com

A catalogue record for this book is available from the British Library

Designed by Wolfgang Homola

Typeset by Davidson Publishing Solutions, Glasgow

Printed and bound in Italy by LEGO Spa, Lavis (Trento)

### Acknowledgements

We would like to thank those authors and publishers who kindly gave permission for copyright material to be used in the Collins corpus. We would also like to thank Times Newspapers Ltd for providing valuable data.

All rights reserved. No part of this book may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without the prior permission in writing of the Publisher. This book is sold subject to the conditions that it shall not, by way of trade or otherwise, be lent, re-sold, hired out or otherwise circulated without the publisher's prior consent in any form of binding or cover other than that in which it is published and without a similar condition including this condition being imposed on the subsequent purchaser.

Entered words that we have reason to believe constitute trademarks have been designated as such. However, neither the presence nor absence of such designation should be regarded as affecting the legal status of any trademark.

HarperCollins does not warrant that www.collinsdictionary.com, www.collinslanguage.com or any other website mentioned in this title will be provided uninterrupted, that any website will be error free, that defects will be corrected, or that the website or the server that makes it available are free of viruses or bugs. For full terms and conditions please refer to the site terms provided on the website.

### About the type

This dictionary is typeset in CollinsFedra, a special version of the Fedra family of types designed by Peter Bil'ak. CollinsFedra has been customized especially for Collins dictionaries; it includes both sans serif (for headwords) and serif (entries) versions, in several different weights. Its large x-height, its open 'eye', and its basis in the tradition of humanist letterforms make CollinsFedra both familiar and easy to read at small sizes. It has been designed to use the minimum space without sacrificing legibility, as well as including a number of characters and signs that are specific to dictionary typography. Its companion phonetic type is the first of its kind to be drawn according to the same principles as the regular typeface, rather than assembled from rotated and reflected characters from other types. Peter Bil'ak (born 1973, Slovakia) is a graphic and type designer living in the Netherlands. He is the author of two books, Illegibility and Transparency. As well as the Fedra family, he has designed several other typefaces including Eureka. His typotheque.com website has become a focal point for research and debate around contemporary type design.

This material may be protected by Copyright law (Title 17 U.S. Code)

**sage**<sup>2</sup> (seid3) n 1a perennial Mediterranean plant, Salvia officinalis, having grey-green leaves and purple, blue, or white flowers: family Lamiaceae (labiates) 2 the leaves of this plant, used in cooking for flavouring 3 short for sagebrush

**ETYMOLOGY** C14: from Old French saulge, from Latin salvia, from salvus safe, in good health (from the curative properties attributed to the plant)

sagebrush ('seɪdʒ,brʌʃ) n any of several aromatic plants of the genus Artemisia, esp A. tridentata, a shrub of W North America, having silver-green leaves and large clusters of small white flowers: family Asteraceae (composites)

sage Derby n See Derby2 (sense 4)

sage grouse n a large North American grouse, Centrocercus urophasianus, the males of which perform elaborate courtship displays

 ETYMOLOGY C19: so named because it lives among, and eats, SACEBRUSH

**saggar** or **sagger** ('sægə) *n* a clay box in which fragile ceramic wares are placed for protection during firing

**SETYMOLOGY** C17: perhaps alteration of SAFEGUARD **sagging moment** *n* a bending moment that produces concave bending at the middle of a simple supported beam. Also called: **positive bending moment** 

Saghalien (sə'gɑ:ljən) n a variant of Sakhalin Sagitta (sə'gɪtə) n, Latin genitive Sagittae (sə'gɪti:) a small constellation in the N hemisphere lying between Cygnus and Aquila and crossed by the Milky Way

**ETYMOLOGY** C16: from Latin, literally: an arrow **sagittal** ('sædʒttal') *adj* 1 resembling an arrow; straight 2 of or relating to the sagittal suture 3 situated in a plane parallel to the sagittal suture > 'sagittally ady

**sagittal suture** n a serrated line on the top of the skull that marks the junction of the two parietal bones

Sagittarius (,sæd31'teəriəs) n, Latin genitive Sagittarii (,sæd31'teəri,ai) 1 astronomy a large conspicuous zodiacal constellation in the S hemisphere lying between Scorpius and Capricornus on the ecliptic and crossed by the Milky Way and containing the galactic centre 2 Also called: the Archer astrology a the ninth sign of the zodiac, symbol ₹, having a mutable fire classification and ruled by the planet Jupiter. The sun is in this sign between Nov 22 and Dec 21 b a person born when the sun is in this sign ▷ adj 3 astrology born under or characteristic of Sagittarius ▶ Also (senses 2b, 3): Sagittarian (,sæd31'teəriən)

**©ETYMOLOGY** C14: from Latin: an archer, from sagitta an arrow

**sagittate** ('sædʒɪˌteɪt) or **sagittiform** (sə'dʒɪtɪˌfɔːm, 'sædʒ-) adj (esp of leaves) shaped like the head of an arrow

**ETYMOLOGY** C18: from New Latin sagittātus, from Latin sagitta arrow

**Sago** ('seigəu) n a starchy cereal obtained from the powdered pith of a sago palm, used for puddings and as a thickening agent

●ETYMOLOGY C16: from Malay sāgū

**sago grass** n Austral a tall tough grass, Paspalidum globoideum, grown as forage for cattle

**sago palm** n 1 any of various tropical Asian palm trees, esp any of the genera *Metroxylon*, *Arenga*, and *Caryota*, the trunks of which yield sago 2 any of several palmlike cycads that yield sago, esp *Cycas revoluta* 

Saguaro (səˈgwɑːrəʊ, səˈwɑː-) or sahuaro (səˈwɑːrəʊ) n, pl -ros a giant cactus, Carnegiea gigantea, of desert regions of Arizona, S California, and Mexico, having white nocturnal flowers and edible red pulpy fruits

 ETYMOLOGY Mexican Spanish, variant of sahuaro, an Indian name

Saguenay (,sægə'nei) n a river in SE Canada in S Quebec, rising as the Péribonca River on the central plateau and flowing south, then east to the St Lawrence. Length: 764 km (475 miles) Sagunto (Spanish sa' unto) n an industrial town in E Spain, near Valencia: allied to Rome and made a heroic resistance to the Carthaginian attack led by Hannibal (219–218 BC). Pop: 58287 (2003 est). Ancient name: Saguntum (sa'gu:ntəm)

Sahaptin (so:'hæptin), Sahaptan (so:'hæptən), or Sahaptian (so:'hæptiən) n 1 (pl-tins, -tans, -tians or -tin, -tan, -tian) a member of a North American Indian people of Oregon and Washington, including the Nez Percé 2 the language of this people > Ancient name: Shahaptin (b'hæptin)

Sahara (səˈhɑ:rə) n a desert in N Africa, extending from the Atlantic to the Red Sea and from the Mediterranean to central Mali, Niger, Chad, and the Sudan: the largest desert in the world, occupying over a quarter of Africa; rises to over 3300 m (11000 ft) in the central mountain system of the Ahaggar and Tibesti massifs; large reserves of iron ore, oil, and natural gas. Area: 9100000 sq km (3500000 sq miles). Average annual rainfall: less than 254 mm (10 in.). Highest recorded temperature: 58°C (136.4°F)

Saharan (sə'ho:rən) n 1a group of languages spoken in parts of Chad and adjacent countries, now generally regarded as forming a branch of the Nilo-Saharan family ▷ adj 2 relating to or belonging to this group of languages 3 of or relating to the Sahara

**sahib** ('sɑ:hɪb) or **saheb** ('sɑ:hɛb) n (in India) a form of address or title placed after a man's name or designation, used as a mark of respect

**• ЕТУМОLOGY** C17: from Urdu, from Arabic çāhib, literally: friend

Sahitya Akademi (sa:ˈhɪtjə əˈkɑːdəmɪ) n a body set up by the Government of India for cultivating literature in Indian languages and in English

**saice** (sais) *n* a variant spelling of **syce** 

said¹ (sed) adj 1 (prenominal) (in contracts, pleadings, etc) named or mentioned previously; aforesaid ▷ vb 2 the past tense and past participle of say¹ said² ('so:id) n a variant of sayyid

Saida ('sa:idə) n a port in SW Lebanon, on the Mediterranean: on the site of ancient Sidon; terminal of the Trans-Arabian pipeline from Saudi Arabia. Pop: 150 000 (2005 est)

saiga ('saiga) n either of two antelopes, Saiga tatarica or S. mongolica, of the plains of central Asia, having an enlarged slightly elongated nose

• ETYMOLOGY C19: from Russian

Saigon (sar'gon) n the former name (until 1976) of Ho Chi Minh City

sail (seil) n lan area of fabric, usually Terylene or nylon (formerly canvas), with fittings for holding it in any suitable position to catch the wind, used for propelling certain kinds of vessels, esp over water 2 a voyage on such a vessel: a sail down the river 3 a vessel with sails or such vessels collectively: to travel by sail; we raised seven sail in the northeast 4 a ship's sails collectively 5 something resembling a sail in shape, position, or function, such as the part of a windmill that is turned by the wind or the part of a Portuguese man-of-war that projects above the water 6 the conning tower of a submarine 7 in sail having the sail set 8 make sail a to run up the sail or to run up more sail b to begin a voyage 9 set sail a to embark on a voyage by ship b to hoist sail 10 under sail a with sail hoisted b under way > vb (mainly intr) 11 to travel in a boat or ship: we sailed to Le Havre 12 to begin a voyage; set sail; we sail at 5 o'clock 13 (of a vessel) to move over the water: the liner is sailing to the Caribbean 14 (tr) to manoeuvre or navigate a vessel: he sailed the schooner up the channel 15 (tr) to sail over: she sailed the Atlantic single-handed 16 (often foll by over, through, etc) to move fast or effortlessly: we sailed through customs; the ball sailed over the fence 17 to move along smoothly; glide 18 (often foll by in or into) informal a to begin (something) with vigour b to make an attack (on) violently with words or physical force > 'sailable adj > 'sailless adj

**ETYMOLOGY** Old English segl; related to Old Frisian seil, Old Norse segl, German Segel

**sailboard** ('seil,bo:d) n the craft used for windsurfing, consisting of a moulded board like a surfboard, to which a mast bearing a single sail is attached by a swivel joint **sailboarding** ('seil,bo:din) *n* another name for windsurfing

sailcloth ('Seil,kloo) n 1 any of various fabrics from which sails are made 2 a lighter cloth used for clothing, etc

**sailer** ('serlə) *n* a vessel, esp one equipped with sails, with specified sailing characteristics: *a good sailer* 

sailfish ('senl,fij) n, pl-fish or -fishes 1 any of several large scombroid game fishes of the genus Istiophorus, such as l. albicans (Atlantic sailfish), of warm and tropical seas: family Istiophoridae. They have an elongated upper jaw and a long sail-like dorsal fin 2 another name for basking shark

sailing ('seilin) n 1 the practice, art, or technique of sailing a vessel 2 a method of navigating a vessel: rhumb-line sailing 3 an instance of a vessel's leaving a port: scheduled for a midnight sailing

sailing boat or esp US and Canadian sailboat ('seilbout) n a boat propelled chiefly by sail sailing ship n a large sailing vessel

sailor ('seila) n 1 any member of a ship's crew, esp one below the rank of officer 2 a person who sails, esp with reference to the likelihood of his becoming seasick: a good sailor 3 short for sailor hat, sailor suit > 'sailorly adj

**sailor hat** *n* a hat with a flat round crown and fairly broad brim that is rolled upwards

sailor's-choice n any of various small percoid fishes of American coastal regions of the Atlantic, esp the grunt Haemulon parra and the pinfish

sailor suit π a child's suit, usually navy and white, with a collar that is squared off at the back like a sailor's

**sailplane** ('seil,plein) n a high-performance glider **sain** (sein) vb (tr) archaic to make the sign of the cross over so as to bless or protect from evil or sin **ETYMOLOGY** Old English segnian, from Latin signare to sign (with the cross)

sainfoin ('sænfoin) n a Eurasian perennial leguminous plant, Onobrychis viciifolia, widely grown as a forage crop, having pale pink flowers and curved pods

 ETYMOLOGY C17: from French, from Medieval Latin sānum faenum wholesome hay, referring to its former use as a medicine

saint (seint; unstressed sont) n 1 a person who after death is formally recognized by a Christian Church, esp the Roman Catholic Church, as having attained, through holy deeds or behaviour, a specially exalted place in heaven and the right to veneration 2 a person of exceptional holiness or goodness 3 (plural) Bible the collective body of those who are righteous in God's sight ▷ wb 4 (tr) to canonize; recognize formally as a saint ▷ 'saintdom n ▷ 'saintless adj ▷ 'saintlike adj

**ETYMOLOGY** c12: from Old French, from Latin sanctus holy, from sancīre to hallow

Saint Agnes's Eve n the night of Jan 20, when according to tradition a woman can discover the identity of her future husband by performing certain rites. Abbreviation: St Agnes's Eve

Saint Albans ('5:lbənz) n a city in SE England, in W Hertfordshire: founded in 948 AD around the Benedictine abbey first built in Saxon times on the site of the martyrdom (about 303 AD) of St Alban; present abbey built in 1077; Roman ruins. Pop: 82 429 (2001). Abbreviation: St Albans. Latin name:

**Saint Andrews** *n* a city in E Scotland, in Fife on the North Sea: the oldest university in Scotland (1411); famous golf links. Pop: 14209 (2001). Abbreviation: **St Andrews** 

Saint Andrew's Cross n 1 a diagonal cross with equal arms 2 a white diagonal cross on a blue ground, Abbreviation: St Andrew's Cross

**ETYMOLOGY** c18: so called because Saint Andrew, one of the twelve apostles of Jesus, is reputed to have been crucified on a cross of this shape

Saint Anthony's Cross n another name for tau cross. Abbreviation: St Anthony's Cross

Saint Anthony's fire n pathol another name for ergotism or erysipelas. Abbreviation: St Anthony's fire

# EXHIBIT 11

# Case 1:21-cv-01015-JLH Document 174-2 Filed 03/20/23 Page 73 of 318 PageID #: 7174

#### HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VYONDYS 53 safely and effectively. See full prescribing information for VYONDYS 53.

VYONDYS 53 (golodirsen) injection, for intravenous use Initial U.S. Approval: 2019

#### - RECENT MAJOR CHANGES-

Dosage and Administration (2.1, 2.2, 2.3, 2.4) Warnings and Precautions (5.2)

#### 2/2021 2/2021

#### -INDICATIONS AND USAGE-

VYONDYS 53 is an antisense oligonucleotide indicated for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the *DMD* gene that is amenable to exon 53 skipping. This indication is approved under accelerated approval based on an increase in dystrophin production in skeletal muscle observed in patients treated with VYONDYS 53. Continued approval for this indication may be contingent upon verification of a clinical benefit in confirmatory trials. (1)

#### DOSAGE AND ADMINISTRATION-

- Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VYONDYS 53 (2.1)
- 30 milligrams per kilogram once weekly (2.2)
- Administer as an intravenous infusion over 35 to 60 minutes via an inline 0.2 micron filter (2.2, 2.4)
- Dilution required prior to administration (2.3)

#### -DOSAGE FORMS AND STRENGTHS

Injection: 100 mg/2 mL (50 mg/mL) in a single-dose vial (3)

#### -CONTRAINDICATIONS-

None (4)

#### -----WARNINGS AND PRECAUTIONS-----

- Hypersensitivity Reactions: Hypersensitivity reactions, including rash, pyrexia, pruritus, urticaria, dermatitis, and skin exfoliation have occurred in patients who were treated with VYONDYS 53. If a hypersensitivity reaction occurs, institute appropriate medical treatment and consider slowing the infusion or interrupting the VYONDYS 53 therapy. (2.3, 5.1)
- Kidney Toxicity: Based on animal data, may cause kidney toxicity.
   Kidney function should be monitored; creatinine may not be a reliable measure of renal function in DMD patients. (5.2, 13.2)

#### ADVERSE REACTIONS-

The most common adverse reactions (incidence ≥20% and higher than placebo) were headache, pyrexia, fall, abdominal pain, nasopharyngitis, cough, vomiting, and nausea. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact Sarepta Therapeutics, Inc. at 1-888-SAREPTA (1-888-727-3782) or FDA at 1-800-FDA-1088 or <a href="www.fda.gov/medwatch">www.fda.gov/medwatch</a>.

See 17 for PATIENT COUNSELING INFORMATION

Revised: 2/2021

#### FULL PRESCRIBING INFORMATION: CONTENTS\*

- 1 INDICATIONS AND USAGE
- 2 DOSAGE AND ADMINISTRATION
  - 2.1 Monitoring to Assess Safety
  - 2.2 Dosing Information
  - 2.3 Preparation Instructions
  - 2.4 Administration Instructions
- 3 DOSAGE FORMS AND STRENGTHS
- 5 WARNINGS AND PRECAUTIONS
- 5.1 Hypersensitivity Reactions
  - 5.2 Kidney Toxicity
- 6 ADVERSE REACTIONS
  - 6.1 Clinical Trials Experience
- B USE IN SPECIFIC POPULATIONS
  - 8.1 Pregnancy
  - 8.2 Lactation
  - 8.4 Pediatric Use

- 8.5 Geriatric Use
- 8.6 Patients with Renal Impairment
- 11 DESCRIPTION
- 12 CLINICAL PHARMACOLOGY
  - 12.1 Mechanism of Action
  - 12.2 Pharmacodynamics
  - 12.3 Pharmacokinetics
- 3 NONCLINICAL TOXICOLOGY
  - 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility
  - 13.2 Animal Toxicology and/or Pharmacology
- 14 CLINICAL STUDIES
- 16 HOW SUPPLIED/STORAGE AND HANDLING
  - 16.1 How Supplied
  - 16.2 Storage and Handling
- 17 PATIENT COUNSELING INFORMATION

<sup>\*</sup>Sections or subsections omitted from the full prescribing information are not listed.

#### **FULL PRESCRIBING INFORMATION**

#### 1 INDICATIONS AND USAGE

VYONDYS 53 is indicated for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the *DMD* gene that is amenable to exon 53 skipping. This indication is approved under accelerated approval based on an increase in dystrophin production in skeletal muscle observed in patients treated with VYONDYS 53 [see Clinical Studies (14)]. Continued approval for this indication may be contingent upon verification of a clinical benefit in confirmatory trials.

# 2 DOSAGE AND ADMINISTRATION

# 2.1 Monitoring to Assess Safety

Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VYONDYS 53. Consider measurement of glomerular filtration rate prior to initiation of VYONDYS 53. Monitoring for kidney toxicity during treatment is recommended. Obtain the urine samples prior to infusion of VYONDYS 53 or at least 48 hours after the most recent infusion [see Warnings and Precautions (5.2)].

# **2.2 Dosing Information**

The recommended dosage of VYONDYS 53 is 30 milligrams per kilogram administered once weekly as a 35 to 60-minute intravenous infusion via an in-line 0.2 micron filter.

If a dose of VYONDYS 53 is missed, it may be administered as soon as possible after the scheduled dose.

# 2.3 Preparation Instructions

VYONDYS 53 is supplied in single-dose vials as a preservative-free concentrated solution that requires dilution prior to administration. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. Use aseptic technique.

- a. Calculate the total dose of VYONDYS 53 to be administered based on the patient's weight and the recommended dose of 30 milligrams per kilogram. Determine the volume of VYONDYS 53 needed and the correct number of vials to supply the full calculated dose.
- b. Allow the vials to warm to room temperature. Mix the contents of each vial by gently inverting 2 or 3 times. Do not shake.
- c. Visually inspect each vial of VYONDYS 53. The solution is a clear to slightly opalescent, colorless liquid, and may contain trace amounts of small, white to off-white amorphous particles. Do not use if the solution in the vials is cloudy, discolored or

- contains extraneous particulate matter other than trace amounts of small, white to offwhite amorphous particles.
- d. With a syringe fitted with a 21-gauge or smaller bore non-coring needle, withdraw the calculated volume of VYONDYS 53 from the appropriate number of vials.
- e. Dilute the withdrawn VYONDYS 53 in 0.9% Sodium Chloride Injection, USP, to make a total volume of 100 to 150 mL. Gently invert 2 to 3 times to mix. Do not shake. Visually inspect the diluted solution. Do not use if the solution is cloudy, discolored or contains extraneous particulate matter other than trace amounts of small, white to off-white amorphous particles.
- f. Administer the diluted solution via an in-line 0.2 micron filter.
- g. VYONDYS 53 contains no preservatives and should be administered immediately after dilution. Complete infusion of diluted VYONDYS 53 within 4 hours of dilution. If immediate use is not possible, the diluted product may be stored for up to 24 hours at 2°C to 8°C (36°F to 46°F). Do not freeze. Discard unused VYONDYS 53.

#### 2.4 Administration Instructions

Application of a topical anesthetic cream to the infusion site prior to administration of VYONDYS 53 may be considered.

VYONDYS 53 is administered via intravenous infusion. Flush the intravenous access line with 0.9% Sodium Chloride Injection, USP, prior to and after infusion.

Infuse the diluted VYONDYS 53 over 35 to 60 minutes via an in-line 0.2 micron filter. Do not mix other medications with VYONDYS 53 or infuse other medications concomitantly via the same intravenous access line with VYONDYS 53.

If a hypersensitivity reaction occurs, consider slowing the infusion or interrupting the VYONDYS 53 therapy [see Warnings and Precautions (5.1) and Adverse Reactions (6.1)].

#### 3 DOSAGE FORMS AND STRENGTHS

VYONDYS 53 is a clear to slightly opalescent, colorless liquid, and may contain trace amounts of small, white to off-white amorphous particles, and available as:

• Injection: 100 mg/2 mL (50 mg/mL) solution in a single-dose vial

#### 4 CONTRAINDICATIONS

None.

#### 5 WARNINGS AND PRECAUTIONS

# 5.1 Hypersensitivity Reactions

Hypersensitivity reactions, including rash, pyrexia, pruritus, urticaria, dermatitis, and skin exfoliation have occurred in VYONDYS 53-treated patients, some requiring treatment. If a hypersensitivity reaction occurs, institute appropriate medical treatment and consider slowing the infusion or interrupting the VYONDYS 53 therapy [see Dosage and Administration (2.4)].

# 5.2 Kidney Toxicity

Kidney toxicity was observed in animals who received golodirsen [see Use in Specific Populations (8.4)]. Although kidney toxicity was not observed in the clinical studies with VYONDYS 53, the clinical experience with VYONDYS 53 is limited, and kidney toxicity, including potentially fatal glomerulonephritis, has been observed after administration of some antisense oligonucleotides. Kidney function should be monitored in patients taking VYONDYS 53. Because of the effect of reduced skeletal muscle mass on creatinine measurements, creatinine may not be a reliable measure of kidney function in DMD patients. Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VYONDYS 53. Consider also measuring glomerular filtration rate using an exogenous filtration marker before starting VYONDYS 53. During treatment, monitor urine dipstick every month, and serum cystatin C and urine protein-to-creatinine ratio every three months. Only urine expected to be free of excreted VYONDYS 53 should be used for monitoring of urine protein. Urine obtained on the day of VYONDYS 53 infusion prior to the infusion, or urine obtained at least 48 hours after the most recent infusion, may be used. Alternatively, use a laboratory test that does not use the reagent pyrogallol red, as this reagent has the potential to cross react with any VYONDYS 53 that is excreted in the urine and thus lead to a false positive result for urine protein.

If a persistent increase in serum cystatin C or proteinuria is detected, refer to a pediatric nephrologist for further evaluation.

#### 6 ADVERSE REACTIONS

The following serious adverse reactions are described below and elsewhere in the labeling:

• Hypersensitivity Reactions [see Warnings and Precautions (5.1)]

# **6.1** Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

In the VYONDYS 53 clinical development program, 58 patients received at least one intravenous dose of VYONDYS 53, ranging between 4 mg/kg (0.13 times the recommended dosage) and 30 mg/kg (the recommended dosage). All patients were male and had genetically confirmed Duchenne muscular dystrophy. Age at study entry was 6 to 13 years. Most (86%) patients were Caucasian.

VYONDYS 53 was studied in 2 double-blind, placebo-controlled studies.

In Study 1 Part 1, patients were randomized to receive once-weekly intravenous infusions of VYONDYS 53 (n=8) in four increasing dose levels from 4 mg/kg to 30 mg/kg or placebo (n=4), for at least 2 weeks at each level. All patients who participated in Study 1 Part 1 (n=12) were continued into Study 1 Part 2, an open-label extension, during which they received VYONDYS 53 at a dose of 30 mg/kg IV once weekly [see Clinical Studies (14)].

In Study 2, patients received VYONDYS 53 (n=33) 30 mg/kg or placebo (n=17) IV once weekly for up to 96 weeks, after which all patients received VYONDYS 53 at a dose of 30 mg/kg.

Adverse reactions observed in at least 20% of treated patients in the placebo-controlled sections of Studies 1 and 2 are shown in Table 1.

Table 1: Adverse Reactions That Occurred in At Least 20% of VYONDYS 53-Treated Patients and at a Rate Greater than Placebo in Studies 1 and 2

	VYONDYS 53	Placebo
Adverse Reaction	(N=41)	(N=21)
	%	%
Headache	41	10
Pyrexia	41	14
Fall	29	19
Abdominal pain	27	10
Nasopharyngitis	27	14
Cough	27	19
Vomiting	27	19
Nausea	20	10

Other adverse reactions that occurred at a frequency greater than 5% of VYONDYS 53-treated patients and at a greater frequency than placebo were: administration site pain, back pain, pain, diarrhea, dizziness, ligament sprain, contusion, influenza, oropharyngeal pain, rhinitis, skin abrasion, ear infection, seasonal allergy, tachycardia, catheter site related reaction, constipation, and fracture.

Hypersensitivity reactions have occurred in patients treated with VYONDYS 53 [see Warnings and Precautions (5.1)].

## 8 USE IN SPECIFIC POPULATIONS

# 8.1 Pregnancy

#### **Risk Summary**

There are no human or animal data available to assess the use of VYONDYS 53 during pregnancy. In the U.S. general population, major birth defects occur in 2 to 4% and miscarriage occurs in 15 to 20% of clinically recognized pregnancies.

#### 8.2 Lactation

#### Risk Summary

There are no human or animal data to assess the effect of VYONDYS 53 on milk production, the presence of golodirsen in milk, or the effects of VYONDYS 53 on the breastfed infant.

The developmental and health benefits of breastfeeding should be considered along with the mother's clinical need for VYONDYS 53 and any potential adverse effects on the breastfed infant from VYONDYS 53 or from the underlying maternal condition.

#### 8.4 Pediatric Use

VYONDYS 53 is indicated for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the *DMD* gene that is amenable to exon 53 skipping, including pediatric patients [see Clinical Studies (14)].

Intravenous administration of golodirsen (0, 100, 300, or 900 mg/kg) to juvenile male rats once weekly for 10 weeks (postnatal days 14 to 77) did not result in postnatal developmental (e.g., neurobehavioral, immune function, or male reproductive) toxicity. However, at the highest dose tested (900 mg/kg/week), golodirsen resulted in the death of animals because of renal impairment or failure. In surviving animals (including one animal at the lowest dose tested), there was a dose-dependent increase in the incidence and severity of renal tubular effects (including degeneration/regeneration, fibrosis, vacuolation, and dilatation), which correlated with changes in clinical pathology parameters, reflecting a dose-dependent impairment of renal function. In addition, decreases in bone area, mineral content, and mineral density were observed at the highest dose tested (900 mg/kg week) but with no effect on bone growth. A noeffect dose for renal toxicity was not identified; the lowest dose tested (100 mg/kg/week) was associated with plasma exposures (AUC) approximately 2.5 times that in humans at the recommended human dose of 30 mg/kg/week.

#### 8.5 Geriatric Use

DMD is largely a disease of children and young adults; therefore, there is no geriatric experience with VYONDYS 53.

# **8.6** Patients with Renal Impairment

Renal clearance of golodirsen is reduced in non-DMD adults with renal impairment, based on estimated glomerular filtration rate calculated using the Modification of Diet and Renal Disease (MDRD) equation [see Clinical Pharmacology (12.3)]. However, because of the effect of reduced skeletal muscle mass on creatinine measurements in DMD patients, no specific dosage adjustment can be recommended for DMD patients with renal impairment based on estimated glomerular filtration rate. Patients with known renal function impairment should be closely monitored during treatment with VYONDYS 53.

## 11 DESCRIPTION

VYONDYS 53 (golodirsen) injection is a sterile, aqueous, preservative-free, concentrated solution for dilution prior to intravenous administration. VYONDYS 53 is a clear to slightly opalescent, colorless liquid, and may contain trace amounts of small, white to off-white amorphous particles. VYONDYS 53 is supplied in single-dose vials containing 100 mg golodirsen (50 mg/mL). VYONDYS 53 is formulated as an isotonic phosphate buffered saline solution with an osmolality of 260 to 320 mOSM and a pH of 7.5. Each milliliter of VYONDYS 53 contains: 50 mg golodirsen; 0.2 mg potassium chloride; 0.2 mg potassium phosphate monobasic; 8 mg sodium chloride; and 1.14 mg sodium phosphate dibasic, anhydrous, in water for injection. The product may contain hydrochloric acid or sodium hydroxide to adjust pH.

Golodirsen is an antisense oligonucleotide of the phosphorodiamidate morpholino oligomer (PMO) subclass. PMOs are synthetic molecules in which the five-membered ribofuranosyl rings found in natural DNA and RNA are replaced by a six-membered morpholino ring. Each morpholino ring is linked through an uncharged phosphorodiamidate moiety rather than the negatively charged phosphate linkage that is present in natural DNA and RNA. Each phosphorodiamidate morpholino subunit contains one of the heterocyclic bases found in DNA (adenine, cytosine, guanine, or thymine). Golodirsen contains 25 linked subunits. The sequence of bases from the 5' end to 3' end is GTTGCCTCCGGTTCTGAAGGTGTTC. The molecular formula of golodirsen is C<sub>305</sub>H<sub>481</sub>N<sub>138</sub>O<sub>112</sub>P<sub>25</sub> and the molecular weight is 8647.28 daltons.

The structure of golodirsen is:

# 12 CLINICAL PHARMACOLOGY

## **12.1** Mechanism of Action

Golodirsen is designed to bind to exon 53 of dystrophin pre-mRNA resulting in exclusion of this exon during mRNA processing in patients with genetic mutations that are amenable to exon 53 skipping. Exon 53 skipping is intended to allow for production of an internally truncated dystrophin protein in patients with genetic mutations that are amenable to exon 53 skipping [see Clinical Studies (14)].

# 12.2 Pharmacodynamics

After treatment with VYONDYS 53, all patients evaluated (n=25) in Study 1 Part 2 [see Clinical Studies (14)] had an increase in skipping of exon 53 demonstrated by reverse transcription polymerase chain reaction (RT-PCR), compared to baseline.

In Study 1 Part 2 [see Clinical Studies (14)], dystrophin levels as assessed by the Sarepta western blot assay increased from 0.10% (SD 0.07) of normal at baseline to 1.02% (SD 1.03) of normal after 48 weeks of treatment with VYONDYS 53. The mean change from baseline in

dystrophin after 48 weeks of treatment with VYONDYS 53 was 0.92% (SD 1.01) of normal levels (p<0.001); the median change from baseline was 0.88%. This increase in dystrophin protein expression positively correlated with the level of exon skipping. Dystrophin levels assessed by western blot can be meaningfully influenced by differences in sample processing, analytical technique, reference materials, and quantitation methodologies. Therefore, comparing dystrophin results from different assay protocols will require a standardized reference material and additional bridging studies.

Correct localization of truncated dystrophin to the sarcolemma in muscle fibers of patients treated with golodirsen was demonstrated by immunofluorescence staining.

#### 12.3 Pharmacokinetics

The pharmacokinetics of golodirsen was evaluated in DMD patients following administration of intravenous doses ranging from 4 mg/kg/week to 30 mg/kg/week (i.e., recommended dosage). Golodirsen exposure increased proportionally with dose, with minimal accumulation with onceweekly dosing. Inter-subject variability (as %CV) for C<sub>max</sub> and AUC ranged from 38% to 72%, and 34% to 44%, respectively.

#### **Distribution**

Steady-state volume of distribution was similar between DMD patients and healthy subjects. The mean golodirsen steady-state volume of distribution was 668 mL/kg (%CV=32.3) at a dose of 30 mg/kg. Golodirsen plasma protein binding ranged from 33% to 39% and is not concentration dependent.

#### Elimination

Golodirsen elimination half-life (SD) was 3.4~(0.6) hours, and plasma clearance was 346~mL/hr/kg at the 30~mg/kg dose.

#### Metabolism

Golodirsen is metabolically stable. No metabolites were detected in plasma or urine.

#### Excretion

Golodirsen is mostly excreted unchanged in the urine. The elimination half-life  $(t_{1/2})$  was 3.4 hours.

#### **Specific Populations**

Age:

The pharmacokinetics of golodirsen have been evaluated in male pediatric DMD patients. There is no experience with the use of VYONDYS 53 in DMD patients 65 years of age or older.

Sex:

Sex effects have not been evaluated; VYONDYS 53 has not been studied in female patients.

Race:

The potential impact of race is not known because 92% of the patients in studies were Caucasians.

#### Patients with Renal Impairment:

The effect of renal impairment on the pharmacokinetics of golodirsen was evaluated in non-DMD subjects aged 41 to 65 years with Stage 2 chronic kidney disease (CKD) (n=8, estimated glomerular filtration rate (eGFR) ≥60 and <90 mL/min/1.73 m²) or Stage 3 CKD (n=8, eGFR ≥30 and <60 mL/min/1.73 m²) and matched healthy subjects (n=8, eGFR ≥90 mL/min/1.73 m²). Subjects received a single 30 mg/kg IV dose of golodirsen.

In subjects with Stage 2 or Stage 3 CKD, exposure (AUC) increased approximately 1.2-fold and 1.9-fold, respectively. There was no change in the  $C_{max}$  in subjects with Stage 2 CKD; in subjects with Stage 3 CKD, there was a 1.2-fold increase in  $C_{max}$  compared with subjects with normal renal function. The effect of Stage 4 or Stage 5 CKD on golodirsen pharmacokinetics and safety has not been studied.

Estimated GFR values derived from MDRD equations and the threshold definitions for various CKD stages in otherwise healthy adults would not be generalizable to pediatric patients with DMD. Therefore, no specific dosage adjustment can be recommended for patients with renal impairment [see Use in Specific Populations (8.6)].

## Patients with Hepatic Impairment:

VYONDYS 53 has not been studied in patients with hepatic impairment.

#### **Drug Interaction Studies**

Golodirsen did not inhibit CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP3A4/5 *in vitro*. Golodirsen was a weak inducer of CYP1A2 and did not induce CYP2B6 or CYP3A4. Golodirsen was not metabolized by human hepatic microsomes and was not a substrate or strong inhibitor of any of the key human drug transporters tested (OAT1, OAT3, OCT2, OATP1B1, MATE1, P-gp, BCRP, and MRP2, OATP1B3 and MATE2-K). Based on *in vitro* data, golodirsen has a low potential for drug-drug interactions in humans.

#### 13 NONCLINICAL TOXICOLOGY

# 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

#### Carcinogenesis

Carcinogenicity studies have not been conducted with golodirsen.

#### Mutagenesis

Golodirsen was negative in *in vitro* (bacterial reverse mutation and chromosomal aberration in CHO cells) and *in vivo* (mouse bone marrow micronucleus) assays.

#### Impairment of Fertility

Fertility studies in animals were not conducted with golodirsen. No effects of golodirsen on the male reproductive system were observed following weekly subcutaneous administration (0, 120,

300, or 600 mg/kg to male mice or weekly intravenous administration (0, 80, 200, or 400 mg/kg) to male monkeys. Plasma exposure (AUC) at the highest doses tested in mouse and monkey are approximately 10 and 45 times that in humans at the recommended weekly intravenous dose of 30 mg/kg.

# 13.2 Animal Toxicology and/or Pharmacology

Kidney toxicity was observed in studies in male mice and rats; findings in urinary bladder were observed in male mice.

In male mice, golodirsen was administered weekly for 12 weeks by intravenous injection (0, 12, 120, or 960 mg/kg) or for 26 weeks by subcutaneous injection (0, 120, 300, or 600 mg/kg). In the 12-week study, microscopic findings in kidney (tubular dilatation, basophilic or eosinophilic casts, vacuolation), correlated with increases in serum markers of renal function (e.g., urea nitrogen, creatinine), were observed primarily at the highest dose tested; hypertrophy of the transitional epithelium of the ureter or urinary bladder was observed at all doses. In the 26-week study, renal tubular degeneration and degeneration of the transitional epithelium of the urinary bladder were observed at all doses.

In male rats, intravenous administration of golodirsen (0, 60, 100, 300, or 600 mg/kg) weekly for 13 weeks resulted in tubular degeneration at all but the lowest dose tested; at the high dose, the microscopic changes were accompanied by increases in serum urea nitrogen.

In male monkeys, intravenous administration of golodirsen (0, 80, 200, or 400 mg/kg) weekly for 39 weeks resulted in microscopic changes in kidney (basophilia, dilatation, or mononuclear cell infiltration) at all doses, which correlated with increases in serum markers of renal function (urea nitrogen, creatinine) at the highest dose tested.

## 14 CLINICAL STUDIES

The effect of VYONDYS 53 on dystrophin production was evaluated in one study in DMD patients with a confirmed mutation of the *DMD* gene that is amenable to exon 53 skipping (Study 1; NCT02310906).

Study 1 Part 1 was a double-blind, placebo-controlled, dose-titration study in 12 DMD patients. Patients were randomized 2:1 to receive VYONDYS 53 or matching placebo. VYONDYS 53-treated patients received four escalating dose levels, ranging from 4 mg/kg/week (less than the recommended dosage) to 30 mg/kg/week, by intravenous infusion for 2 weeks at each dose level.

Study 1 Part 2 was a 168-week, open-label study assessing the efficacy and safety of VYONDYS 53 at a dose of 30 mg/kg/week in the 12 patients enrolled in Part 1, plus 13 additional treatment-naive patients with DMD amenable to exon 53 skipping. At study entry (either in Part 1 or Part 2), patients had a median age of 8 years and were on a stable dose of corticosteroids for at least 6 months. Efficacy was assessed based on change from baseline in the dystrophin protein level (measured as % of the dystrophin level in healthy subjects, i.e., % of normal) at Week 48 of Part 2. Muscle biopsies were obtained at baseline prior to treatment and at Week 48 of Part 2 in all VYONDYS 53-treated patients (n=25), and were analyzed for dystrophin protein level by Sarepta western blot. Mean dystrophin levels increased from 0.10% (SD 0.07) of normal at

baseline to 1.02% (SD 1.03) of normal by Week 48 of Study 1 Part 2, with a mean change in dystrophin of 0.92% (SD 1.01) of normal levels (p<0.001); the median change from baseline was 0.88%.

Individual patient dystrophin levels from Study 1 are shown in Table 2.

Table 2: Dystrophin Expression Sarepta Western Blot by Individual Patient From Study 1

	Sarepta Western Blot % Normal Dystrophin			Sarepta Western Blot % Normal Dystrophin			
Patient Number	Baseline	Part 2 Week 48	Change from baseline	Patient number	Baseline	Part 2 Week 48	Change from baseline
1	0.08	0.09	0.01	14	0.22	0.28	0.06
2	0.11	0.11	0.01	15	0.14	0.21	0.07
3	0.21	0.22	0.01	16	0.05	0.42	0.37
4	0.05	0.12	0.08	17	0.07	1.03	0.97
5	0.03	0.12	0.09	18	0.02	1.57	1.55
6	0.06	0.14	0.09	19	0.12	1.17	1.05
7	0.12	0.37	0.25	20	0.03	1.72	1.69
8	0.11	1.06	0.95	21	0.11	1.77	1.66
9	0.06	0.54	0.48	22	0.31	4.30	3.99
10	0.05	0.97	0.92	23	0.11	0.36	0.25
11	0.06	1.55	1.49	24	0.03	0.91	0.88
12	0.07	1.91	1.84	25	0.07	1.29	1.22
13	0.10	3.25	3.15				

## 16 HOW SUPPLIED/STORAGE AND HANDLING

# 16.1 How Supplied

VYONDYS 53 injection is supplied in single dose vials. The solution is a clear to slightly opalescent, colorless liquid, and may contain trace amounts of small, white to off-white amorphous particles.

• Single-dose vials containing 100 mg/2mL (50 mg/mL)

NDC 60923-465-02

# 16.2 Storage and Handling

Store VYONDYS 53 at 2°C to 8°C (36°F to 46°F). Do not freeze. Store in original carton until ready for use to protect from light.

#### 17 PATIENT COUNSELING INFORMATION

#### Hypersensitivity Reactions

Advise patients and/or caregivers that hypersensitivity reactions, including rash, pyrexia, pruritus, urticaria, dermatitis, and skin exfoliation have occurred in patients who were treated with VYONDYS 53. Instruct them to seek immediate medical care should they experience signs and symptoms of hypersensitivity [see Warnings and Precautions (5.1)].

#### **Kidney Toxicity**

Inform patients nephrotoxicity has occurred with drugs similar to VYONDYS 53. Advise patients of the importance of monitoring for kidney toxicity by their healthcare providers during treatment with VYONDYS 53 [see Warnings and Precautions (5.2)].

Manufactured for: Sarepta Therapeutics, Inc. Cambridge, MA 02142 USA

SAREPTA, SAREPTA THERAPEUTICS, and VYONDYS are trademarks of Sarepta Therapeutics, Inc. registered in the U.S. Patent and Trademark Office and may be registered in various other jurisdictions. VYONDYS 53, and the Vyondys 53 logo are trademarks of Sarepta Therapeutics, Inc.



# EXHIBIT 12

From: Miller, Zachary D.

To: Lee, Yoonjin; Williamson, Amanda S.; Dudash, Amy M.

Cc: Blumenfeld, Jack; Dellinger, Megan E.; Lipsey, Charles; Raich, William; Flibbert, Michael; Lipton, Alissa;

McCorquindale, J. Derek, Kozikowski, John, Clay, Aaron, NS District Court

**Subject:** Re: Proposed Joint Constructions **Date:** Sunday, January 1, 2023 7:38:23 PM

#### **EXTERNAL** Email:

#### Yoonjin,

I believe you misunderstood our position with respect to the first term. Our position is not that the term of the '361 Patent permits nucleobase additions, substitutions, or modifications. Rather, our position is that NS's construction requiring "a nucleotide sequence that is [or that is identical to] the specific sequence of SEQ ID NO: 57" already makes clear that the nucleobases of the claimed AON are the same as those of SEQ ID NO: 57. The additional language proposed by Sarepta is unnecessary, redundant, and runs contrary to claim construction principles, so should not be included.

With respect to the terms of the '322 Patent, NS believes that Sarepta's use of the term "directly" is improper, as it restricts the use of other reagents in the claimed chemical reactions. NS's proposal removes that restriction given that the claims use "comprising" language. In addition, while each step requires the use of at least two specific reagents to form a specific result (e.g., step e) requires reacting Compound 3 and a deprotecting agent to make Compound 4), method claims generally do not require a specific order to the individual steps, as long as each specific step is met. Accordingly, NS's proposal removes the language in Sarepta's construction that calls for a specific step order.

We look forward to discussing with you tomorrow.

Thanks,

Zach

#### Zachary D. Miller

Morgan, Lewis & Bockius LLP

110 North Wacker Drive | Chicago, IL 60606-1511

Direct: +1.312.324.1706 | Main: +1.312.324.1000 | Fax: +1.312.324.1001 | Mobile: +1.937.581.0258

zachary.miller@morganlewis.com | www.morganlewis.com

Assistant: Millie McAllister | +1.312.324.1488 | mildred.mcallister@morganlewis.com



From: Lee, Yoonjin < Yoonjin.Lee@finnegan.com> Sent: Thursday, December 29, 2022 5:38:05 PM

**To:** Williamson, Amanda S. <amanda.williamson@morganlewis.com>; Miller, Zachary D. <zachary.miller@morganlewis.com>; Dudash, Amy M. <amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack <JBlumenfeld@morrisnichols.com>; Dellinger, Megan E.

<mdellinger@morrisnichols.com>; Lipsey, Charles <charles.lipsey@finnegan.com>; Raich, William

<William.Raich@finnegan.com>; Flibbert, Michael <michael.flibbert@finnegan.com>; Lipton, Alissa <Alissa.Lipton@finnegan.com>; McCorquindale, J. Derek <Derek.McCorquindale@finnegan.com>; Kozikowski, John <John.Kozikowski@finnegan.com>; Clay, Aaron <Aaron.Clay@finnegan.com>; NS District Court <NSDistrictCourt@morganlewis.com>

Subject: RE: Proposed Joint Constructions

#### [EXTERNAL EMAIL]

Amanda,

We are available at 11 AM ET on Monday. Please send us the dial-in information.

To facilitate our discussions, please provide before our meet and confer any authority supporting NS's position that the term of the '361 patent permits nucleobase additions, substitutions, or modifications. Similarly, as requested in our correspondence dated December 29, please provide before our meet and confer NS's explanation on whether and how in NS's view, Sarepta's proposed constructions for the terms of the '322 patent differ from those proposed by NS on December 20.

Regards, Yoonjin

#### Yoonjin Lee, Ph.D.

Associate

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP 901 New York Avenue, NW, Washington, DC 20001-4413 +1 202 408 4332 | fax: +1 202 408 4400 | <a href="mailto:yoonjin.lee@finnegan.com">yoonjin.lee@finnegan.com</a> | <a href="mailto:www.finnegan.com">www.finnegan.com</a> | <a hr

# FINNEGAN

From: Williamson, Amanda S. <amanda.williamson@morganlewis.com>

Sent: Thursday, December 29, 2022 3:22 PM

**To:** Lee, Yoonjin < Yoonjin.Lee@finnegan.com>; Miller, Zachary D.

<zachary.miller@morganlewis.com>; Dudash, Amy M. <amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack <JBlumenfeld@morrisnichols.com>; Dellinger, Megan E.

<mdellinger@morrisnichols.com>; Lipsey, Charles <charles.lipsey@finnegan.com>; Raich, William <William.Raich@finnegan.com>; Flibbert, Michael <michael.flibbert@finnegan.com>; Lipton, Alissa <Alissa.Lipton@finnegan.com>; McCorquindale, J. Derek <Derek.McCorquindale@finnegan.com>; Kozikowski, John <John.Kozikowski@finnegan.com>; Clay, Aaron <Aaron.Clay@finnegan.com>; NS District Court <NSDistrictCourt@morganlewis.com>

Subject: RE: Proposed Joint Constructions

#### **EXTERNAL** Email:

Lee,

We are available at 11am ET or 2pm ET on Monday to meet and confer. If these times will not work,

please propose an alternative that works for your team.

We do not agree to your alternative construction for the '361 patent as it adds impermissible negative limitations, i.e. "with no nucleobase additions, substitutions, or modifications." For clarity, should we need to brief these terms, NS will rely on the constructions provided as its plain and ordinary meaning.

Best regards, Amanda

#### **Amanda S. Williamson**

Morgan, Lewis & Bockius LLP

110 North Wacker Drive, Suite 2800 | Chicago, IL 60606-1511

Direct: +1.312.324.1450 | Main: +1.312.324.1000 | Fax: +1.312.324.1001

amanda.williamson@morganlewis.com | www.morganlewis.com

Assistant: Melissa C. Martier | +1.312.324.1161 | melissa.martier@morganlewis.com

From: Lee, Yoonjin < <a href="mailto:Yoonjin.Lee@finnegan.com">Yoonjin.Lee@finnegan.com</a>>
Sent: Thursday, December 29, 2022 8:10 AM

**To:** Miller, Zachary D. <<u>zachary.miller@morganlewis.com</u>>; Dudash, Amy M.

<amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Flibbert, Michael <<u>michael.flibbert@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>; Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS

District Court < <a href="mailto:NSDistrictCourt@morganlewis.com">NSDistrictCourt@morganlewis.com</a> <a href="mailto:Subject: RE: Proposed Joint Constructions">Subject: RE: Proposed Joint Constructions</a>

Judject. NE. 110 posed some construction

[EXTERNAL EMAIL] Counsel,

We have considered NS's proposed joint constructions of the terms from the NS patents but do not understand NS's reasoning for departing from Sarepta's initial proposed constructions.

Starting with the term from the '361 patent, Sarepta believes that its initial proposed construction will be beneficial to the jury as it explains that no base changes are allowed. To that end, we propose the following hybrid construction:

Plain and ordinary meaning – i.e., Antisense oligomer with a nucleotide sequence that is <u>identical to</u> the <del>specific</del> nucleotide sequence of SEQ ID NO: 57 (guugccuccg guucugaagg uguuc) <u>with no nucleobase additions, substitutions, or modifications</u>.

Please let us know if you agree with this alternative construction.

Turning to the terms from the '322 patent, NS proposes construing those terms in accordance with

their plain and ordinary meaning and yet also proposes: (1) deleting "directly," (2) changing "which results in" to "in order to form," (3) omitting the references to steps d) and e), and (4) omitting "Step f) must occur after step e)" from Sarepta's initial proposed constructions. Please explain whether and how in NS's view, Sarepta's proposed constructions for those terms differ from those proposed by NS on December 20. Once we have a better understanding of NS's positions, we will reconsider NS's proposal and/or propose potential alternative constructions, if any.

We can be available to meet and confer early next week, if NS believes it is necessary.

Regards, Yoonjin

#### Yoonjin Lee, Ph.D.

#### Associate

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP 901 New York Avenue, NW, Washington, DC 20001-4413 +1 202 408 4332 | fax: +1 202 408 4400 | yoonjin.lee@finnegan.com | www.finnegan.com

# FINNEGAN

From: Lee, Yoonjin

Sent: Wednesday, December 21, 2022 9:27 AM

**To:** Miller, Zachary D. <<u>zachary.miller@morganlewis.com</u>>; Flibbert, Michael

<michael.flibbert@finnegan.com>; Dudash, Amy M. <amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William

<William.Raich@finnegan.com>; Lipton, Alissa <Alissa.Lipton@finnegan.com>; McCorquindale, J.

Derek < Derek. McCorquindale@finnegan.com >; Kozikowski, John < John. Kozikowski@finnegan.com >;

Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>>

**Subject:** RE: Proposed Joint Constructions

Counsel,

We acknowledge the receipt of your email and proposal. We will get back to you in due course.

Regards, Yoonjin

#### Yoonjin Lee, Ph.D.

#### Associate

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP 901 New York Avenue, NW, Washington, DC 20001-4413

+1 202 408 4332 | fax: +1 202 408 4400 | yoonjin.lee@finnegan.com | www.finnegan.com



From: Miller, Zachary D. <<u>zachary.miller@morganlewis.com</u>>

**Sent:** Tuesday, December 20, 2022 10:14 AM

**To:** Lee, Yoonjin < <a href="mailto:Yoonjin.Lee@finnegan.com">Yoonjin.Lee@finnegan.com</a>; Flibbert, Michael < <a href="mailto:michael.flibbert@finnegan.com">michael.flibbert@finnegan.com</a>;

Dudash, Amy M. <a href="mailto:amy.dudash@morganlewis.com">amy.dudash@morganlewis.com</a>>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>; Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>>

**Subject:** Proposed Joint Constructions

#### **EXTERNAL** Email:

#### Counsel,

I apologize for being unable to provide the below proposed joint constructions prior to last week's submission. However, we believe that it is worthwhile to have a discussion regarding the terms in the NS Patents prior to briefing, to determine if we can avoid burdening the Court with unnecessary argument. Below are our proposed joint constructions for the terms in the NS Patents.

Patent (Claims)	<u>Term</u>	Sarepta's Position	Proposed Joint Construction
NS Patent - U.S. Patent No. 9,708,361 (1, 3-7)	"antisense oligomer consisting of the nucleotide sequence of SEQ ID NO: 57"	Antisense oligomer having the sequence of SEQ ID NO: 57 (guugccuccg guucugaagg uguuc) with no nucleobase additions, substitutions or modifications thereof.	Plain and ordinary meaning – i.e., Antisense oligomer with a nucleotide sequence that is the specific nucleotide sequence of SEQ ID NO: 57 (guugccuccg guucugaagg uguuc)
NS Patent - U.S. Patent No. 10,683,322 (Claims 1-10)	"e) reacting said Compound 3 with a deprotecting agent to form Compound 4"	Plain and ordinary meaning, i.e., chemically reacting a deprotecting agent directly with Compound 3 of step d), which results in Compound 4.	Plain and ordinary meaning – i.e., chemically reacting Compound 3 with a deprotecting agent, in order to form Compound 4
NS Patent - U.S. Patent No. 10,683,322 (Claims 1-10)	"f) reacting Compound 4 with an acid to form said oligomer" or "f) reacting said Compound 4 with an	Plain and ordinary meaning, i.e., chemically reacting an acid directly with Compound 4 of step e), which results in the oligomer or the	Plain and ordinary meaning – i.e., chemically reacting Compound 4 with an acid, in order to form the oligomer [or the PMO]

# Case 1:21-cv-01015-JLH Document 174-2 Filed 03/20/23 Page 92 of 318 PageID #: 7193

acid to form said PMO"	PMO.	
	Step f) must occur after step e).	

Please let us know if Sarepta is willing to agree to these proposed joint constructions, or if Sarepta has any proposed edits for NS to consider. If it would be helpful to have a discussion, we are available on Thursday or Friday of this week to meet and confer. Thanks,

Zach

#### Zachary D. Miller

#### Morgan, Lewis & Bockius LLP

110 North Wacker Drive | Chicago, IL 60606-1511

Direct: +1.312.324.1706 | Main: +1.312.324.1000 | Fax: +1.312.324.1001 | Mobile: +1.937.581.0258

<u>zachary.miller@morganlewis.com</u> | <u>www.morganlewis.com</u>

Assistant: Millie McAllister | +1.312.324.1488 | mildred.mcallister@morganlewis.com

From: Lee, Yoonjin < <a href="mailto:Yoonjin.Lee@finnegan.com">Yoonjin.Lee@finnegan.com</a>>
Sent: Thursday, December 15, 2022 1:53 PM

**To:** Miller, Zachary D. <<u>zachary.miller@morganlewis.com</u>>; Flibbert, Michael

<michael.flibbert@finnegan.com>; Dudash, Amy M. <amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>; Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>>

**Subject:** RE: Claim Construction Exchange

[EXTERNAL EMAIL] Counsel,

Thank you for providing us with the combined Claim Construction Charts, the current pleading, and the exhibits. We propose one edit to the cover pleading as shown below:

S	U.S. Appl. No. 15/274,772 Response to Final OA dated Nov.
	16, 2017 Excerpts of the File History of U.S. Application No.
	15/274,772

Please confirm that this is acceptable to NS. Once we receive your confirmation, we will separately follow up on your signatory question.

Regards, Yoonjin

#### Yoonjin Lee, Ph.D.

#### Associate

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP 901 New York Avenue, NW, Washington, DC 20001-4413 +1 202 408 4332 | fax: +1 202 408 4400 | yoonjin.lee@finnegan.com | www.finnegan.com

# FINNEGAN

From: Miller, Zachary D. <<u>zachary.miller@morganlewis.com</u>>

Sent: Thursday, December 15, 2022 12:29 PM

**To:** Lee, Yoonjin < <a href="mailto:Yoonjin.Lee@finnegan.com">Yoonjin.Lee@finnegan.com</a>>; Flibbert, Michael < <a href="mailto:michael.flibbert@finnegan.com">michael.flibbert@finnegan.com</a>>;

Dudash, Amy M. <amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

< <u>William.Raich@finnegan.com</u>>; Lipton, Alissa < <u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J.

Derek < <u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John < <u>John.Kozikowski@finnegan.com</u>>;

Clay, Aaron < <u>Aaron.Clay@finnegan.com</u>>; NS District Court < <u>NSDistrictCourt@morganlewis.com</u>>

**Subject:** RE: Claim Construction Exchange

#### **EXTERNAL** Email:

Counsel,

We have combined the exchanged exhibits to create the final exhibits for filing and will send those via FTP shortly. Attached are the final versions of the cover pleading and claim charts. Please let us know if you have any further edits prior to filing, and if no further edits, please confirm we can add your signature. Thanks,

Zach

#### Zachary D. Miller

#### Morgan, Lewis & Bockius LLP

110 North Wacker Drive | Chicago, IL 60606-1511

Direct: +1.312.324.1706 | Main: +1.312.324.1000 | Fax: +1.312.324.1001 | Mobile: +1.937.581.0258

zachary.miller@morganlewis.com | www.morganlewis.com

Assistant: Millie McAllister | +1.312.324.1488 | mildred.mcallister@morganlewis.com

From: Miller, Zachary D.

Sent: Thursday, December 15, 2022 7:58 AM

**To:** 'Lee, Yoonjin' < <u>Yoonjin.Lee@finnegan.com</u>>; Flibbert, Michael

<michael.flibbert@finnegan.com>; Dudash, Amy M. <amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<mdellinger@morrisnichols.com>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William

<<u>Milliam.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>; Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>> **Subject:** Claim Construction Exchange

Counsel,

Attached is an updated version of the draft cover pleading accepting your edits. Also attached are versions of the claim charts with NS's positions filled in. We will be sending the intrinsic evidence cited by NS via FTP shortly. Thanks,

Zach

## Zachary D. Miller

#### Morgan, Lewis & Bockius LLP

110 North Wacker Drive | Chicago, IL 60606-1511

Direct: +1.312.324.1706 | Main: +1.312.324.1000 | Fax: +1.312.324.1001 | Mobile: +1.937.581.0258

zachary.miller@morganlewis.com | www.morganlewis.com

Assistant: Millie McAllister | +1.312.324.1488 | mildred.mcallister@morganlewis.com

From: Lee, Yoonjin < <a href="mailto:Yoonjin.Lee@finnegan.com">Yoonjin.Lee@finnegan.com</a>>
Sent: Tuesday, December 13, 2022 6:29 PM

**To:** Miller, Zachary D. <<u>zachary.miller@morganlewis.com</u>>; Flibbert, Michael

<michael.flibbert@finnegan.com>; Dudash, Amy M. <amv.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>; Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>>

**Subject:** RE: Joint technical tutorial

[EXTERNAL EMAIL] Zach,

Thank you for agreeing to the exchange time of 9 AM on Thursday. We also appreciate your confirmation that NS will handle the filing on that day and your proposed draft cover pleading. We proposed a few minor edits to the cover pleading in the attached. Please let us know if the revision is acceptable to NS. We also reserve the right to make further revisions, should NS make additional edits.

We generally agree with your proposed exhibit numbering scheme. To the extent that the parties intend to rely on any other intrinsic evidence, we propose assigning them sequentially after Exhibit Q. For the UWA patents and their file wrappers, we intend to rely on the following produced versions:

The '851 Patent	SRT-VYDS-00002641-756
The '590 Patent	SRT-VYDS-00002757-869
The '827 Patent	SRT-VYDS-00002870-983
Excerpts of the File History of the '851 Patent	SRT-VYDS-00002984-5241
Excerpts of the File History of the '590 Patent	SRT-VYDS-00005242-5992
Excerpts of the File History of the '827 Patent	SRT-VYDS-00005993-6843

We look forward to receiving NS's proposed joint constructions for the currently disputed terms of the NS patents.

Regards, Yoonjin

#### Yoonjin Lee, Ph.D.

Associate

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP 901 New York Avenue, NW, Washington, DC 20001-4413

+1 202 408 4332 | fax: +1 202 408 4400 | yoonjin.lee@finnegan.com | www.finnegan.com

# FINNEGAN

From: Miller, Zachary D. < <a href="mailto:zachary.miller@morganlewis.com">zachary.miller@morganlewis.com</a>>

Sent: Tuesday, December 13, 2022 1:27 PM

To: Lee, Yoonjin < <a href="mailto:Yoonjin.Lee@finnegan.com">Yoonjin.Lee@finnegan.com</a>; Flibbert, Michael <a href="mailto:Michael.flibbert@finnegan.com">Michael.flibbert@finnegan.com</a>;

Dudash, Amy M. <a href="mailto:amy.dudash@morganlewis.com">amy.dudash@morganlewis.com</a>>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>;

Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>>

Subject: RE: Joint technical tutorial

#### **EXTERNAL** Email:

Yoonjin,

Further to my email below, attached is a draft cover pleading. Thanks,

Zach

## Zachary D. Miller Morgan, Lewis & Bockius LLP

110 North Wacker Drive | Chicago, IL 60606-1511

Direct: +1.312.324.1706 | Main: +1.312.324.1000 | Fax: +1.312.324.1001 | Mobile: +1.937.581.0258

#### <u>zachary.miller@morganlewis.com</u> | <u>www.morganlewis.com</u>

Assistant: Millie McAllister | +1.312.324.1488 | mildred.mcallister@morganlewis.com

**From:** Miller, Zachary D.

Sent: Tuesday, December 13, 2022 10:00 AM

**To:** 'Lee, Yoonjin' < <u>Yoonjin.Lee@finnegan.com</u>>; Flibbert, Michael

<michael.flibbert@finnegan.com>; Dudash, Amy M. <amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>; Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>>

**Subject:** RE: Joint technical tutorial

Yoonjin,

Thank you for the comments. We agree to exchange charts and intrinsic evidence at 9AM ET on Thursday. As for numbering the intrinsic evidence, we propose the following:

Exhibit	Description of Exhibit
С	The '361 Patent
D	The '092 Patent
E	The '461 Patent
F	The '106 Patent
G	The '741 Patent
Н	The '217 Patent
I	The '322 Patent
J	Excerpts of the File History of the '361 Patent
<b>K</b> Excerpts of the File History of the '322 Patent	
L	The '851 Patent
M	The '590 Patent
N	The '827 Patent
0	Excerpts of the File History of the '851 Patent
Р	Excerpts of the File History of the '590 Patent
Q	Excerpts of the File History of the '827 Patent

For the excerpts of each of the file histories, we propose ordering those by Bates number. Note, that for the NS Patents, we presently intend to cite to the prosecution histories of the '361 Patent and '322 Patent; if Sarepta intends to cite to other file histories, we will add those excerpts accordingly. In order to coordinate, we also believe it makes sense for the parties to cite to the same produced versions of the file histories to avoid attaching multiple versions of the same document. For the NS Patents, we intend to cite to beginning Bates No. NS00000477 for the File History of the '361 Patent and beginning Bates No. NS00034978 for the File History of the '322

Patent. We'd appreciate it if you could identify the beginning Bates numbers for the file histories of the UWA Patents that you intend to use.

In addition, we will file the joint chart and will provide a draft cover pleading shortly.

We also agree that Sarepta's proposed construction can be first for the UWA Patents. We are working to provide proposed joint constructions for the disputed terms of the NS Patents as soon as possible and plan to do so no later than tomorrow morning. Thanks,

7ach

#### Zachary D. Miller

#### Morgan, Lewis & Bockius LLP

110 North Wacker Drive | Chicago, IL 60606-1511

Direct: +1.312.324.1706 | Main: +1.312.324.1000 | Fax: +1.312.324.1001 | Mobile: +1.937.581.0258

zachary.miller@morganlewis.com | www.morganlewis.com

Assistant: Millie McAllister | +1.312.324.1488 | mildred.mcallister@morganlewis.com

From: Lee, Yoonjin < <a href="mailto:Yoonjin.Lee@finnegan.com">Yoonjin.Lee@finnegan.com</a>>
Sent: Monday, December 12, 2022 3:00 PM

**To:** Miller, Zachary D. <<u>zachary.miller@morganlewis.com</u>>; Flibbert, Michael

<michael.flibbert@finnegan.com>; Dudash, Amy M. <amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>; Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>>

**Subject:** RE: Joint technical tutorial

[EXTERNAL EMAIL] Zach,

Thank you for sending us the shell Joint Claim Construction Charts. We agree with your proposed order of the terms from the UWA patents, i.e., the entire phrase first and then the three subphrases within. In addition, we propose the following:

#### • Exchange Deadline:

We propose moving the deadline for the simultaneous exchange to 9 AM ET on
 Thursday (Dec. 15) so that any issues that may arise upon exchange can be addressed in a timely manner. Please confirm that NS agrees with the change.

# • Filing Logistics:

• We understand that NS will handle the filing of the Joint Claim Construction Charts. Please confirm that our understanding is correct.

- We believe that that Charts should be filed with a cover pleading. Please provide us with a draft for our review at your earliest convenience.
- The Scheduling Order requires the parties to submit copies of the patents at issue and any portion of intrinsic evidence cited in the Charts. We therefore propose exchanging copies of those materials when the parties exchange the Charts. Please let us know if you have a proposal for numbering those materials.

#### • Claim Charts:

- For the Chart that includes the terms from the UWA patents, we believe that Sarepta's proposed constructions and supportive intrinsic evidence should be listed first. For the simultaneous exchange, we will update that Chart accordingly.
- During our meet and confer, you indicated that NS would provide proposed joint
  constructions for the terms from the NS patents to Sarepta. Please provide us with
  those alternative constructions at your earliest convenience. Ideally, we would like to
  receive them by COB tomorrow so that the parties can potentially avoid burdening
  the Court with extraneous filings.

Given the approaching deadline, we would appreciate your response by noon ET tomorrow.

Regards, Yoonjin

#### Yoonjin Lee, Ph.D.

Associate

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP 901 New York Avenue, NW, Washington, DC 20001-4413 +1 202 408 4332 | fax: +1 202 408 4400 | yoonjin.lee@finnegan.com | www.finnegan.com

# FINNEGAN

From: Miller, Zachary D. < <a href="mailto:zachary.miller@morganlewis.com">zachary.miller@morganlewis.com</a>>

**Sent:** Monday, December 12, 2022 12:29 PM

**To:** Flibbert, Michael <<u>michael.flibbert@finnegan.com</u>>; Dudash, Amy M.

<amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack < <u>IBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>;

Clay, Aaron < <u>Aaron.Clay@finnegan.com</u>>; NS District Court < <u>NSDistrictCourt@morganlewis.com</u>>

Subject: RE: Joint technical tutorial

#### **EXTERNAL** Email:

Mike,

Thank you for the helpful discussion regarding claim construction last Friday. With respect to the claim term order for the UWA Patents, we propose the following:

- The entire phrase: "antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA" be listed as term 1;
- The subphrases: "a base sequence," "a target region," and "exon 53 of the human dystrophin pre-mRNA" be listed as sub-terms 1a, 1b, and 1c within term 1 respectively.

We believe that this listing properly considers the terms as they are actually structured in the UWA Patents. Please let us know if Sarepta agrees to this proposal.

We have also attached blank Joint Claim Construction Charts for each of the NS Patents and the UWA Patents. We propose that the parties exchange the filled in charts at noon ET on Thursday, so that they can be finalized for filing. Thanks,

Zach

## Zachary D. Miller Morgan, Lewis & Bockius LLP

110 North Wacker Drive | Chicago, IL 60606-1511

Direct: +1.312.324.1706 | Main: +1.312.324.1000 | Fax: +1.312.324.1001 | Mobile: +1.937.581.0258

zachary.miller@morganlewis.com | www.morganlewis.com

Assistant: Millie McAllister | +1.312.324.1488 | mildred.mcallister@morganlewis.com

**From:** Flibbert, Michael <<u>michael.flibbert@finnegan.com</u>>

Sent: Tuesday, December 6, 2022 4:19 PM

**To:** Dudash, Amy M. <a href="mailto:amy.dudash@morganlewis.com">amy.dudash@morganlewis.com</a>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>; Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>>

**Subject:** RE: Joint technical tutorial

[EXTERNAL EMAIL]

Amy,

We are available on Friday, 12/9 between 11:00 a.m. and 3:00 p.m. to meet and confer on the claim construction issues. Could you please let us know if your team is available then? Thanks.

Best regards,

Mike

Michael J. Flibbert Bio

#### Partner

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP 901 New York Avenue, NW, Washington, DC 20001-4413 +1 202 408 4493 | fax +1 202 408 4400 | michael.flibbert@finnegan.com | www.finnegan.com

# FINNEGAN

From: Flibbert, Michael

Sent: Tuesday, December 6, 2022 3:17 PM

**To:** Dudash, Amy M. <a href="mailto:</a> <a href="mailto:dudash@morganlewis.com">amy.dudash@morganlewis.com</a> >

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>;

Clay, Aaron < <u>Aaron.Clay@finnegan.com</u>>; NS District Court < <u>NSDistrictCourt@morganlewis.com</u>>

**Subject:** RE: Joint technical tutorial

Amy,

In addition to changing the technical tutorial submission date to January 17, we suggest reordering the dates in the chart of relevant dates to place them in chronological order for clarity. Please send us an updated draft for approval.

Regarding NS's proposal to provide a draft technical tutorial in early January, we don't understand why NS couldn't provide the draft much sooner. In the amended scheduling order, NS proposed submitting the joint technical tutorial to the Court on December 8 – two days from now. Presumably, NS has completed at least a draft of the tutorial. To facilitate coordination and avoid duplication of efforts, we would appreciate receiving whatever draft tutorial NS has prepared (with the understanding that NS may continue to refine it).

Best regards, Mike

#### Michael J. Flibbert Bio

Partner

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP 901 New York Avenue, NW, Washington, DC 20001-4413 +1 202 408 4493 | fax +1 202 408 4400 | michael.flibbert@finnegan.com | www.finnegan.com

# FINNEGAN

From: Dudash, Amy M. <amy.dudash@morganlewis.com>

Sent: Tuesday, December 6, 2022 9:00 AM

**To:** Flibbert, Michael < michael.flibbert@finnegan.com >

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>; Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>> **Subject:** RE: Joint technical tutorial

#### **EXTERNAL** Email:

We are amenable to pushing the joint tutorial submission deadline until January 17, 2023. I anticipate we will send over a draft in early January.

#### Amy M. Dudash

Morgan, Lewis & Bockius LLP

1701 Market Street | Philadelphia, PA 19103-2921

Direct: +1.215.963.4861 | Main: +1.215.963.5000 | Fax: +1.215.963.5001 | Mobile: +1.412.901.1678

-and-

1201 N. Market Street, Suite 2201 | Wilmington, DE 19801

Direct: +1.302.574.7293 | Main: +1.302.574.3000 | Fax: +1.302.574.3001

amy.dudash@morganlewis.com | www.morganlewis.com

Assistant: Ethel Kump | +1.215.963.4810 | ethel.kump@morganlewis.com

**From:** Flibbert, Michael < <u>michael.flibbert@finnegan.com</u>>

Sent: Monday, December 5, 2022 2:59 PM

**To:** Dudash, Amy M. <amy.dudash@morganlewis.com>

**Cc:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>; Clay, Aaron <<u>Aaron.Clay@finnegan.com</u>>; NS District Court <<u>NSDistrictCourt@morganlewis.com</u>>

**Subject:** Joint technical tutorial

[EXTERNAL EMAIL]

Hi Amy,

Thanks for your email. FYI, Sarepta's offices will be closed from 12/23/22 through 1/2/23. We propose Tuesday, 1/17/23 for submission of the joint technical tutorial (since Monday, 1/16/23 is a federal holiday). This would still be more than two months before Judge Williams receives the parties' joint claim construction brief on 3/20/23. Please let us know if this proposal is acceptable to NS. Please also let us know when we can expect to receive a draft version of the tutorial.

Best regards,

Mike

Michael J. Flibbert Bio

Partner

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP 901 New York Avenue, NW, Washington, DC 20001-4413 +1 202 408 4493 | fax +1 202 408 4400 | michael.flibbert@finnegan.com | www.finnegan.com

# FINNEGAN

**From:** Dudash, Amy M. <a href="mailto:amy.dudash@morganlewis.com">amy.dudash@morganlewis.com</a>

Sent: Monday, December 5, 2022 11:00 AM

**To:** Blumenfeld, Jack < <u>JBlumenfeld@morrisnichols.com</u>>; Dellinger, Megan E.

<<u>mdellinger@morrisnichols.com</u>>; Lipsey, Charles <<u>charles.lipsey@finnegan.com</u>>; Raich, William <<u>William.Raich@finnegan.com</u>>; Lipton, Alissa <<u>Alissa.Lipton@finnegan.com</u>>; McCorquindale, J. Derek <<u>Derek.McCorquindale@finnegan.com</u>>; Kozikowski, John <<u>John.Kozikowski@finnegan.com</u>>;

Clay, Aaron < <u>Aaron.Clay@finnegan.com</u>>

**Cc:** NS District Court < <a href="mailto:NSDistrictCourt@morganlewis.com">NSDistrictCourt@morganlewis.com</a>>

**Subject:** FW: Activity in Case 1:21-cv-01015-GBW Nippon Shinyaku.,Ltd. v. Sarepta Therapeutics, Inc. Order

#### **EXTERNAL** Email:

Please see attached an updated scheduling order incorporating the dates the Court ordered.

Regarding the submission of the joint claim construction tutorial, as discussed during the meet and confer it may make sense to push that date back slightly. We have put in a placeholder date of December 30, 2022 for submission – let us know if Sarepta is amenable to this date.

Thanks, Amy

#### Amy M. Dudash

Morgan, Lewis & Bockius LLP

1701 Market Street | Philadelphia, PA 19103-2921

Direct: +1.215.963.4861 | Main: +1.215.963.5000 | Fax: +1.215.963.5001 | Mobile: +1.412.901.1678

-and-

1201 N. Market Street, Suite 2201 | Wilmington, DE 19801

Direct: +1.302.574.7293 | Main: +1.302.574.3000 | Fax: +1.302.574.3001

amy.dudash@morganlewis.com | www.morganlewis.com

Assistant: Ethel Kump | +1.215.963.4810 | ethel.kump@morganlewis.com

This e-mail message is intended only for individual(s) to whom it is addressed and may contain information that is privileged, confidential, proprietary, or otherwise exempt from disclosure under applicable law. If you believe you have received this message in error, please advise the sender by return e-mail and delete it from your mailbox. Thank you.

#### DISCLAIMER

This e-mail message is intended only for the personal use of the recipient(s) named above. This message may be an

attorney-client communication and as such privileged and confidential and/or it may include attorney work product. If you are not an intended recipient, you may not review, copy or distribute this message. If you have received this communication in error, please notify us immediately by e-mail and delete the original message.

This e-mail message is intended only for individual(s) to whom it is addressed and may contain information that is privileged, confidential, proprietary, or otherwise exempt from disclosure under applicable law. If you believe you have received this message in error, please advise the sender by return e-mail and delete it from your mailbox. Thank you.

This e-mail message is intended only for individual(s) to whom it is addressed and may contain information that is privileged, confidential, proprietary, or otherwise exempt from disclosure under applicable law. If you believe you have received this message in error, please advise the sender by return e-mail and delete it from your mailbox. Thank you.

This e-mail message is intended only for individual(s) to whom it is addressed and may contain information that is privileged, confidential, proprietary, or otherwise exempt from disclosure under applicable law. If you believe you have received this message in error, please advise the sender by return e-mail and delete it from your mailbox. Thank you.

This e-mail message is intended only for individual(s) to whom it is addressed and may contain information that is privileged, confidential, proprietary, or otherwise exempt from disclosure under applicable law. If you believe you have received this message in error, please advise the sender by return e-mail and delete it from your mailbox. Thank you.

This e-mail message is intended only for individual(s) to whom it is addressed and may contain information that is privileged, confidential, proprietary, or otherwise exempt from disclosure under applicable law. If you believe you have received this message in error, please advise the sender by return e-mail and delete it from your mailbox. Thank you.

This e-mail message is intended only for individual(s) to whom it is addressed and may contain information that is privileged, confidential, proprietary, or otherwise exempt from disclosure under applicable law. If you believe you have received this message in error, please advise the sender by return e-mail and delete it from your mailbox. Thank you.

# EXHIBIT 13

#### The Aldol Reaction: Organocatalysis Approach 2.07

N Mase, Shizuoka University, 3-5-1 Johoku, Naka-ku, Hamamatsu, Shizuoka, Japan Y Hayashi, Tohoku University, Aoba-ku, Sendai, Japan

c 2014 Elsevier Ltd. All rights reserved.

2.07.1	Introduction	274
2.07.2	Enamine Catalysis	276
2.07.2.1	Intramolecular Aldol Reactions in Enamine Catalysis	277
2.07.2.1.1	Cyclization of ketone nucleophiles with ketone electrophiles	277
2.07.2.1.2	Cyclization of ketone nucleophiles with aldehyde electrophiles	280
2.07.2.1.3	Cyclization of aldehyde nucleophiles with ketone electrophiles	282
2.07.2.1.4	Cyclization of aldehyde nucleophiles with aldehyde electrophiles	283
2.07.2.2	Intermolecular Aldol Reactions in Enamine Catalysis	283
2.07.2.2.1	Ketone nucleophiles with ketone electrophiles	284
2.07.2.2.2	Ketone nucleophiles with aldehyde electrophiles	285
2.07.2.2.2.1	Catalyzed by secondary amine derivatives	285
2.07.2.2.2.2	Catalyzed by primary amine derivatives	292
2.07.2.2.2.3	Syn-selective aldol reactions	294
2.07.2.2.3	Aldehyde nucleophiles with ketone electrophiles	296
2.07 2.2.4	Aldehyde nucleophiles with aldehyde electrophiles	297
2.07.2.2.4.1	Aldol reactions of aldehyde nucleophiles	297
2.07.2.2.4.2	Aldol reactions of acetaldehyde nucleophile	305
2.07.2.3	Aldol Reactions in Water, in the Presence of Water, on Water, and by Water	306
2.07.2.3.1	Water as an additive	307
2.07.2.3.2	Water as a solvent	307
2.07.2.4	Multicatalytic Reaction Sequences	312
2.07.2.4.1	Metal-catalyzed and organocatalytic reaction sequences	312
2.07.2.4.2	Organocatalytic and biocatalytic reaction sequences	312
2.07.2.5	Mechanistic Studies	313
2.07.3	Brønsted Acid and Hydrogen-Bond Catalysis	316
2.07.3.1	Intermolecular Aldol Reactions in Brønsted Acid and Hydrogen-Bond Catalysis	317
2.07.3.1.1	Aldol-type reactions of azlactone	317
2.07.3.1.2	Mukaiyama aldol reactions in Brønsted acid catalysis	317
2.07.3.1.3	Direct aldol reactions in Brønsted acid catalysis	319
2.07.3.2	Intramolecular Aldol Reactions in Brønsted Acid Catalysis	320
2.07.4	Brønsted Base Catalysis Including Bifunctional Catalysis	320
2.07.4.1	Aldol Reactions in Brønsted Base Catalysis Including Bifunctional Catalysis	320
2.07.4.2	Aldol-Lactonization Reactions in Nucleophilic Base Catalysis	323
2.07.5	Phase-Transler Catalysis	327
2.07.5.1	Aldol Reactions in Phase-Transfer Catalysis	327
2.07.6	Supported Organocatalysis	328
2.07.6.1	Covalently Supported Organocatalysts	329
2.07.6.2	Noncovalently Supported Organocatalysts	331
2.07.6.3	Supported Organocatalysts in Multiphasic Systems	333
2.07.7	Conclusions	335
References		335

#### Glossarv

Direct aldol reactions Aldol reactions that take place without prior transformation of the carbonyl compound (e.g., aldol reactions using silyl enol ethers as the nucleophilic donor is not a direct aldol reaction). Enamine An unsaturated compound derived from the reaction of an aldehyde or a ketone with a secondary or primary amine followed by loss of H2O. Its general chemical formula is R1R2C=CR3NR42. If one of the nitrogen substituents is H, it is tautomerized to form an imine. endo Aldol reaction Intramolecular carbon-carbon bond formation is endocyclic to the smallest ring

Enolate A reactive intermediate derived from the loss of a proton from the alpha carbon of a carbonyl group. The general formula is R1R2C=CR3O-.

exo Aldol reaction Intramolecular carbon-carbon bond formation is exocyclic to the smallest ring formed. Iminium intermediate A protonated or substituted imine, the general structure of which is  $R^1R^2C = N^+R^3R^4$ .

Transannular aldol reaction Intramolecular carboncarbon bond formation in a large monocyclic ring to produce a bicyclic molecule.

#### 2.07.1 Introduction

274

Two carbonyl compounds (1 and 3) can combine to form  $\beta$ -hydroxycarbonyl compounds 4 with two new continuous stereocenters in the aldol reaction, discovered by Kane in 1838<sup>1</sup> and by Wurtz in 1872.<sup>2</sup> This synthetically and historically important breakthrough reaction is among the most fundamental reactions in organic chemistry; in addition, it is also an essential biological process involved in glycolysis, gluconeogenesis, and the Calvin cycle. When the carbonyl compound 1 bearing  $\alpha$ -hydrogen atoms is treated with base, the nucleophilic enolate 2a and the electrophilic carbonyl compound 3a coincidentally exist in the reaction vessel. These two charged species 2a and 3a directly form a carbon-carbon bond, affording an aldol 4 through an enolate mechanism. The carbonyl compound 1 is converted to enol 2c in the presence of acid. This enol intermediate 2c, which is nucleophilic at the  $\alpha$ -carbon but less reactive than the enolate 2a, can attack the protonated reactive carbonyl compound 3b through an enol mechanism. Another mechanism is called enamine mechanism. Enamine 2b, which is derived by the reaction of the carbonyl compound 1 with a primary or secondary amine followed by loss of H<sub>2</sub>O, reacts with the carbonyl compound 3a to give aldol 4. Enamine 2b is generally more nucleophilic than the enol 2c, but less nucleophilic than enolate 2a (Scheme 1).

$$\begin{array}{c} & & & & & \\ & & & & \\ & & & & \\$$

**Scheme 1** Aldol reactions of carbonyl compounds.

As  $\beta$ -hydroxycarbonyl units are found in naturally occurring compounds and pharmaceuticals, many researchers have performed an asymmetric aldol reaction with frantic efforts. The Mukaiyama aldol reaction is one of the most successful and practical reactions that is the nucleophilic addition of silyl enol ethers 2d to the carbonyl compound 3a, commonly an aldehyde ( $R^3 = H$ ), catalyzed by Lewis acid such as boron trifluoride or titanium chloride, although previous aldol reactions under basic or acidic conditions have accompanied several undesired side reactions such as self-aldol, cross-aldol, aldol condensation, and double aldol condensation. In 1982, the first catalytic asymmetric version of the Mukaiyama aldol reaction through divalent tin enolates with chiral diamine ligands 5 was achieved, and then many research groups proved this methodology extremely versatile (see Chapter 2.09).3 The Mukaiyama aldol reaction is beyond doubt a brilliant triumph in modern synthetic organic chemistry; however, this reaction includes preactivated silyl enol ethers 2d derived from the carbonyl compound 1 with stoichiometric silylation agent and base (Scheme 2, indirect aldol reaction). In addition, silylated wastes are inherently formed. A better process would circumvent a preactivation process in point of atom efficiency, that is, the carbonyl compound 1 directly reacts with the carbonyl compound 3a in the presence of a catalyst. This is generally categorized as 'direct aldol reaction' (Scheme 2). In 1971, intramolecular asymmetric direct aldol reaction of a triketone catalyzed by (S)-proline 6 was independently reported by the Hajos group 4-5 at Hoffmann-La Roche, Inc. and the Wiechert group 4-7 at Schering A.-G. (see also Scheme 4). A quarter century later, the Shibasaki group developed a bifunctional metal-complex catalyst 7, which is type II aldolase mimicking, in the intermolecular direct aldol reaction in 1997.8 In 2000, the List and Barbas group demonstrated the strategic rediscovery of proline's remarkable ability of type I aldolase mimicking intermolecular organocatalytic direct aldol reaction through the study of aldolase and antibody (see also Scheme 19)."

Scheme 2 Indirect and direct aldol reactions.

After these pioneering research on direct aldol reactions, a number of small metal-free organic molecules as organocatalysts in asymmetric bond-forming reactions and domino reactions have been reported. 10-17 Organocatalysts allow for the enantioselective synthesis of molecules that were not readily available by traditional methods. Research in this area has advanced rapidly over the past decade, and the versatility, simplicity, and safety of organocatalytic reactions have been demonstrated. Organocatalytic direct aldol reactions are classified into three activation modes: (1) donor activation, (2) acceptor activation, and (3) bifunctional activation (Figure 1). In donor activation, the carbonyl compounds 1 are converted to nucleophilic enols 8 in the presence of acid, which can then attack the protonated reactive carbonyl electrophiles 3b through an enol mechanism in Brønsted acid catalysis. The second significant activation mode is called the enamine mechanism. Enamines 9 react with carbonyl electrophiles 3a in the presence of a primary or secondary amine to give aldol 4. The third mode of activation is through the enolate mechanism. The nucleophilic enolate 10 is formed by deprotonation of the  $\alpha$ -hydrogen atom of a carbonyl compound 1 through Brønsted base catalysis to yield 10a or in a phase-transfer reaction to yield 10b. The zwitterionic ammonium enolate 11, derived from the addition of tertiary amine or phosphine nucleophilic catalysts to a ketene, reacts with an aldehyde in a tandem aldol-lactonization process to give a  $\beta$ -lactone. Another zwitterionic ammonium enolate 12, derived from the addition of nucleophilic catalyst to the  $\alpha,\beta$ -unsaturated carbonyl compound, can react with carbonyl electrophiles 3, generally called the Morita-Baylis-Hillman reaction. Acceptor activation 13 through Brønsted acid or hydrogen-bond catalysis is a powerful strategy for carbon-carbon bond formation that is similar to Lewis acid catalysis. The combination of donor and acceptor activation 14 is called bifunctional catalysis; in this mechanism, a single organocatalyst simultaneously activates both the substrates.

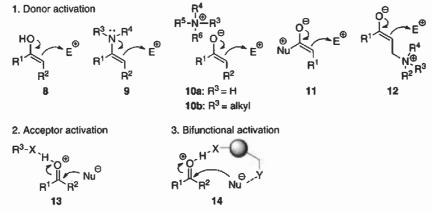


Figure 1 Activation modes and reactive intermediates.

This chapter addresses significant achievements in asymmetric syntheses focused on organocatalytic nucleophilic addition to C=O bonds, namely aldol reactions, covering the literature from 1971 to 2012. <sup>18-24</sup> The description is subdivided based on various classes of catalysis: enamine, Brønsted acid, hydrogen bond, Brønsted base, bifunctional phase transfer, and supported organocatalysis.

#### 2.07.2 Enamine Catalysis

Aldolases are essential, ubiquitous enzymes involved in glycolysis, gluconeogenesis, and the Calvin cycle. They catalyze both carbon-carbon bond formation and cleavage in a stereoselective fashion in the aqueous *in-vivo milieu*. For synthetic chemists, aldolases have become useful tools in modern synthetic organic chemistry, in particular in carbohydrate synthesis; for instance, fructose-1,6-diphosphate aldolase converts p-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate into p-fructose-1,6-diphosphate reversibly *in vitro*. <sup>25-27</sup> Native aldolases are classified into two groups based on the mechanism of nucleophilic activation. Type I aldolases activate nucleophiles through an iminium ion formation step followed by enamine formation (Figure 2, TS-1), whereas type II aldolases activate nucleophiles by forming a zinc enolate (Figure 2, TS-2). Development of a type I aldolase-mimicking antibody catalyst has provided another useful tool in organic chemistry. Aldolase antibodies such as Ab 38C2 and its antipodal antibody siblings catalyze a wide variety of crossed and self-aldol reactions, including intramolecular aldol reactions. <sup>28,29</sup> Type I aldolase-mimicking organocatalysts such as (S)-proline 6 (Figure 2, TS-3) were developed through the active research on aldolases and antibodies in 2000. <sup>9,10</sup>

Figure 2 Transition state models of aldol reaction catalyzed by aldolases or (S)-proline.

Aldol reaction mechanism using aldolase or antibody is intensively investigated (Figure 3, left). <sup>25–27</sup> Type I aldolases activate their donor substrates 15 by the formation of imine 16 with a strictly conserved active site lysine. <sup>229</sup> The imine 16 to the enamine 17 tautomerization generates the nucleophilic species because of raising the energy of its highest occupied molecular orbital (HOMO). Enamine 17 attacks the appropriate face of the electrophilic aldehyde 18 with high stereoselectivity in the active site. The enzyme-bound imine 19 is then hydrolyzed, releasing the corresponding product 20 and aldolase. Similar to the aldolase, an enamine mechanism is commonly considered in (S)-proline-catalyzed direct aldol reaction (Figure 3, right, see also Section 2.07.2.5). The narrow substrate specificity of natural aldolases is vital for life, but not necessarily useful for organic synthesis. Altering the specificity of natural aldolases is relatively difficult and generally requires extensive directed molecular evolution;

Figure 3 Enamine mechanism of aldol reaction catalyzed by aldolase or (S)-proline.

however, it is easy for synthetic chemists to modify a small organic molecule such as (S)-proline 6. Therefore, enamine catalysis based on (S)-proline and its derivatives has been rapidly explored to achieve highly efficient and practical organic syntheses. 30

#### 2.07.2.1 Intramolecular Aldol Reactions in Enamine Catalysis

The intramolecular aldol reaction is an often used approach to the synthesis of cyclic compounds, especially, five- and six-membered rings. <sup>11</sup> The two carbonyl components of the substrate intramolecularly react as both electrophiles and nucleophiles; in addition, they form two different nucleophilic intermediates, namely endo ( $27 \rightarrow 28 \rightarrow 30$ ) and exo ( $27 \rightarrow 29 \rightarrow 31$ ) aldol reactions (Scheme 3). Another type of intramolecular reaction is the *transannular* aldol reaction ( $32 \rightarrow 33 \rightarrow 34$ ) that may be considered *endo* and *exo* simultaneously. Although more than one product may be theoretically formed, excellent chemo-, regio-, and stereo-selectivities have been accomplished in enamine catalysis.

$$R^{1} \xrightarrow{2} \xrightarrow{3} \text{Nn} \xrightarrow{7} \xrightarrow{8} \text{g}^{2}$$

$$27$$

$$28$$

$$endo$$

$$X = R^{1} \text{ or } R^{2}$$

$$X = R^{1} \text{ or } R^{2}$$

$$R^{1} \xrightarrow{1} \text{ or } R^{2}$$

$$X = R^{1} \text{ or } R^{2}$$

$$X = R^{$$

Scheme 3 Intramolecular endo, exo, and transannular aldol reactions.

# 2.07.2.1.1 Cyclization of ketone nucleophiles with ketone electrophiles

The first example of an organocatalytic asymmetric intramolecular 6-endo aldol reaction is the Hajos-Parrish-Eder-Sauer-Wiechert cyclization. In the early 1970s, the Hajos group at Hoffmann-La Roche, Inc.<sup>4,5</sup> and the Wiechert group at Schering A.-C.<sup>6,7</sup> independently reported the enantiogroup-differentiating 6-endo aldol reaction of triketone 35 catalyzed by (S)-proline (Scheme 4).

Scheme 4 Hajos-Parrish-Eder-Sauer-Wiechert reactions.

Hajos' anhydrous condition in dimethylformamide (DMF) affords the aldol product (S)-36 in 100% yield, subsequent acid-promoted dehydration provided (S)-Hajos-Wiechert ketone 37 in 99% yield with 95% ee. In contrast, Wiechert's aqueous conditions in aqueous MeCN/1M HClO<sub>4</sub> directly produce ketone 37 in 87% yield with 84% ee in one-pot operation.

Although the (S)-proline-catalyzed intramolecular aldol reaction of triketone 35 proceeds in excellent yield and enantioselectivity, merely introducing an alkyl group or expanding the ring size led to a decrease in both yield and enantioselectivity when employing (S)-proline 6. Thus, both the Hajos and Eder groups used other amino acids to effect this organocatalytic intramolecular aldol reaction, wherein phenylalanine 39 became the amino acid of choice.<sup>4,6</sup>

Wieland–Miescher ketone analogs 42 have been synthesized using stoichiometric amounts of  $\alpha$ -amino acids under various reaction conditions, as reported by Inomata, Paquette, and coworkers in 2007. Not only phenylalanine 39 but also tryptophan 43, leucine 44, and methionine 45 are efficient catalysts for intramolecular aldol reactions (Scheme 5).

Scheme 5 Synthesis of methylated Wieland-Miescher ketone.

 $\beta$ -Amino acids can be used as catalysts for asymmetric aldol reactions (Scheme 6). When (1*R*,2*S*)-cispentacin 46 (30 mol%) was used as a catalyst for 6-endo aldol reactions of triketone 35, the corresponding bicyclic diketone 37 was obtained in excellent yield with enantioselectivity. The absolute configuration of product 37 is (*R*), whereas (*S*)-isomer 37 is obtained by use of (*S*)-proline 6. Limbach then reported a study of different  $\beta$ -amino acids as aldol catalysts:  $\beta$ <sup>3</sup>-homoproline 47 affords diketone 37 in excellent yields but with low enantioselectivities; however, the use of  $\beta$ -amino acids 48–51 bearing aliphatic side chains results in modest yields but with good enantioselectivities. The absolute configuration of product 37 is (*R*), whereas (*S*)-isomer 37 is obtained by use of (*S*)-proline 6. Limbach then reported a study of different  $\beta$ -amino acids 48–51 bearing aliphatic side chains results in modest yields but with good enantioselectivities.

A one-pot domino Michael-aldol reaction of enone 52 and diketone 53 catalyzed by (S)-proline gives the Wieland-Miescher ketone 56 reported by the Barbas group (Scheme 7).<sup>35</sup> The iminium-based activation of the Michael acceptor 52 (see Chapter 4.03) and the enamine-based activation of triketone 54 are included in the entire Robinson annulation sequence.

Chiral bicyclic diketones such as Hajos-Parrish ketone 37 and Wieland-Miescher ketone 56 are extensively employed for the synthesis of biologically active complex molecules, since Hajos-Parrish-Eder-Sauer-Wiechert intramolecular aldol reaction was reported in 1971. Here, an excellent example of organocatalytic total synthesis of drugs and bioactive natural product is highlighted. Taxol 62 is a highly efficacious anticancer drug used in the treatment of breast, ovarian, lung, bladder, prostate, melanoma, esophageal, as well as other types of solid tumor cancers. Taxol is isolated from the bark of *Taxus brevifolia*, and then many research groups have tried to synthesize this structurally unique molecule through their own strategy. The Danishefsky group employed (S)-proline-catalyzed intramolecular aldol reaction on the first step to install all of the stereochemistry required to reach baccatin III and Taxol in a sequential manner (Scheme 8). <sup>36</sup> As enantiopurity of Wieland-Miescher ketone 56 is readily increased by simple recrystallization in large quantity, the concept of the Wieland-Miescher ketone matrix is favorable for taxol synthesis.

The Agami group reported (S)-proline-catalyzed asymmetric 6-endo aldol reaction of the prochiral diketone 63 in 1984. <sup>37</sup> Cyclization shows a stereoselective si-face attack with moderate enantiomeric excess (equation 2).

# 1. catalyst (20–30 mol%) DMF, r.t., 2–13 days 2. ρ-TsOH, toluene, Δ or H<sub>2</sub>SO<sub>4</sub>, DMF, 95 °C, 3 h 35 Catalysts NHCO<sub>2</sub>H NH<sub>2</sub> NH<sub>2</sub> H CO<sub>2</sub>H NH<sub>2</sub> NH<sub>2</sub> H Oyield 94%, 90% ee (R) yield 99%, 58% ee (R)

yield 64%, 75% ee (R) yield 49%, 78% ee (R) yield 29%, 83% ee (R) yield 54%, 75% ee (R)

Scheme 6 \(\beta\)-Amino acid-catalyzed intramolecular 6-endo aldol reactions.

Scheme 7 One-pot domino Michael-aldol reaction.

Scheme 8 Taxol synthesis from Wieland-Miescher ketone.

280

The enantioselective aldol cyclodehydration of 4-substituted 2,6-heptanediones 65 to cyclohexenones 68 has been a long-term challenge in asymmetric catalysis. In 2008 List and coworkers reported that quinine-derived primary amine 66 in the presence of acetic acid affords cyclic ketone (S)-68 in 94% yield with 90% ee in intramolecular 6-endo aldol reaction of diketone 65, whereas (S)-proline gives the cyclization product 68 in low yields with moderate ee. In addition, the pseudoenantiomeric quinidine-derived primary amine 67 delivers the opposite product, (R)-enantiomer 68, with similar yield and enantioselectivity (Scheme 9). <sup>18</sup>

Scheme 9 Primary amine-catalyzed 6-endo aldol reaction of prochiral diketone.

β-Hydroxy ketones bearing tertiary hydroxy functionality are a very important class of compounds in natural product syntheses; however, ketones are generally poor electrophiles. Furthermore, even if the aldol reaction occurs, dehydration products are mainly obtained as above. The Enders group developed 5-exo aldol reactions of diketone 69 to synthesize cis-selective 3-hydroxy 2,3-dihydrobenzofurans 70 bearing tertiary alcohol (equation 3).

As intramolecular transannular aldol reactions create two new rings and at least two new stereogenic centers in a single process, the corresponding cyclic  $\beta$ -hydroxy ketones 73 are useful for the synthesis of polycyclic natural products. The List group reported that trans-4-fluoro proline 72 is superior to (S)-proline (60% conversion, 54% ee) in the catalysis of this type of reaction with excellent diastereoselectivity and enantioselectivity (Scheme 10). The utility of this reaction has been demonstrated in a total synthesis of (+)-hirustene (74), which is a fungal metabolite first isolated from basidiomycete *Coriolus consors*.

## 2.07.2.1.2 Cyclization of ketone nucleophiles with aldehyde electrophiles

 $\beta$ -Hydroxy ketone bearing secondary hydroxy group is prepared by aldol reactions of ketone nucleophiles with aldehyde electrophiles. In general, reactive  $\alpha$ -unsubstituted aldehydes could not act as an efficient electrophile because of side reactions such as a self-aldol reaction; however, in the following reports chemoselectivities between ketone and aldehyde functional groups are efficiently controlled. In 1981, the Woodward group reported (R)-proline-mediated intramolecular aldol reaction of the racemic ketone-aldehyde 75 in erythromycin synthesis (equation 4). When the racemic ketone-aldehyde 75 is submitted to

Scheme 10 Intramolecular transannular aldol reactions.

intramolecular aldol reaction by use of (S)-proline in benzene/MeOH at 25 °C, aldols 76 obtained are virtually racemic. In contrast, the use of (R)-proline in  $CH_3CN$  at 25 °C leads to a 1:1 mixture of aldols 76 in 70% yield with 36% ee.

In 1987, the Agami group had demonstrated kinetic resolution of racemic ketone-aldehyde 77 through (S)-proline-catalyzed intramolecular 6-ex aldol reaction with moderate enantioselectivity (equation 5).42

In 2005, the Iwabuchi group reported an asymmetric intramolecular 6-exφ aldol reaction of σ-symmetric ketone-aldehyde 79 (Scheme 11). Although (S)-proline itself results in moderate stereoselection, trans-tetrabutylammonium prolinate 80 affords the corresponding (8R)-hydroxybicyclo[3.3.1]nonan-2-one 81 with excellent enantioselectivity at low catalyst loading. Interestingly, opposite enantiopreferences are found in the transformation of 79 to (S)-81 with cis-siloxy proline 82.<sup>43</sup> Enantiopurity of the bicyclic aldol (S)-81 is increased up to 99% ee after recrystallization. A stereocontrolled synthesis of (-)-CP55,940 (83), a potent cannabinoid receptor agonist, has been attained by employing this organocatalytic asymmetric aldol reaction. Furthermore, (+)-juvabione (84), a natural sesquiterpene exhibiting insect juvenile hormone activity, has also been synthesized from the same key compound (S)-81.

Similarly, the six-membered nitrogen-containing ring of the morphan scaffold 87 is formed by an intramolecular 6-exo aldol reaction of the aza-ketone-aldehyde 85 in the presence of the siloxy proline 86 carried out by the Diaba and Bonjoch group, though very few methods are currently available to synthesize enantiopure morphans (equation 6).<sup>46</sup>

**Scheme 11** Intramolecular 6-exo aldol reaction of  $\sigma$ -symmetric ketone aldehyde.

The Enders group has developed (S)-proline-catalyzed 5-exo aldol reactions of the ketone-aldehyde 88 to synthesize cis-selective 3-hydroxy 2,3-dihydrobenzofurans 89 that is a key structure for the synthesis of coumarin natural products (Scheme 12). <sup>39</sup> The diastereo- and enantiopure furans 89 are obtained after simple recrystallization from ethyl acetate/hexane.

Scheme 12 cis-Selective 5-exo aldol reactions of ketone aldehyde.

## 2.07.2.1.3 Cyclization of aidehyde nucleophiles with ketone electrophiles

 $\beta$ -Hydroxy aldehydes bearing tertiary hydroxy group are prepared by aldol reactions of aldehyde nucleophiles with ketone electrophiles. Aldehydes are a more reactive electrophile than ketones; especially electrophilicity of unmodified ketones is very low. Thus, there are little reports on this mode of intramolecular aldol reactions. The List group reported (S)-proline-catalyzed

asymmetric 6-exo aldol reaction of aldehyde-ketone 90. This process provides the substituted cyclohexane 91 with excellent enantioselectivity (equation 7).<sup>47</sup>

(2S,3R)-3-Hydroxy-3-methylproline 93 is an efficient catalyst for the 5-exo aldol reaction of aldehyde-ketone 92 reported by the Hamada group. One-pot reduction of aldol with NaBH<sub>4</sub> affords the N-protected pyrrolidine derivative 94, which is converted to 3-methylproline 93 (Scheme 13).<sup>48</sup>

Scheme 13 Intramolecular 5-exo aldol reaction of aldehyde-ketone.

The trifluoroacetic acid salt of 2-(pyrrolidinylmethyl)pyrrolidine 96 is found to be an effective organocatalyst in an asymmetric intramolecular aldol reaction of aldehyde-ketone 95, affording bicyclo[4.3.0]nonane derivatives 97 with the creation of a quaternary carbon center with high enantioselectivity reported by the Hayashi group (equation 8).<sup>49</sup> In this reaction, the aldehyde and ketone act as nucleophile and electrophile, respectively, a rare reversal of their normal roles.

## 2.07.2.1.4 Cyclization of aldehyde nucleophiles with aldehyde electrophiles

β-Hydroxy aldehyde bearing secondary hydroxy group is prepared by aldol reactions of aldehyde nucleophiles with aldehyde electrophiles. (S)-proline-catalyzed asymmetric 6-exo aldol reaction of dialdehyde 98 was reported by the List group. <sup>47</sup> The process provides trans-substituted cyclohexanes 99a-c in excellent diastereo- and enantioselectivities, though a single substituent at the 4-position has an unfavorable effect on the stereoselectivity of 99d. The meso-dialdehyde is also a good substrate, giving equal amounts of two expected anti-configured aldols 99e with excellent enantioselectivity in asymmetric desymmetrization (Scheme 14).

Intramolecular 6-exo aldol reaction has been used for the total synthesis of (+)-cocaine (102). The Pearson group reported aldol reaction of the meso-dialdehyde 100 with (S)-proline, giving the tropane ring skeleton 101 directly with good enantiomeric excess. (+)-Cocaine (102) is synthesized in 6.5% yield and with 86% ee over 14 linear steps starting from commercially available starting materials (Scheme 15). 50

## 2.07.2.2 Intermolecular Aldol Reactions in Enamine Catalysis

Two unsymmetrical molecules of an aldehyde or a ketone combine to generate aldols, in which the carbonyl compounds 1 and 103 including  $\alpha$ -hydrogen atoms form four possible aldols, that is, two self-aldol products (104a and 104c) and two cross-aldol

Scheme 14 Intramolecular 6-exo aldol reaction of dialdehyde.

Scheme 15 Intramolecular 6-exo aldol reaction of dialdehyde for tropane ring skeleton synthesis.

products (104b and 104d) (Scheme 16). When you need a single aldol product, a highly controlled aldol reaction should be designed. A fundamental challenge of chemoselectivity as well as stereoselectivity has been unsolved in intermolecular organocatalytic direct aldol reactions for a long time; however, the List and Barbas group has reported the first intermolecular aldol reaction of a nucleophilic ketone with an electrophilic aldehyde using (S)-proline as an organocatalyst in 2000 (see Scheme 19).

Scheme 16 Intermolecular self- and cross-addol reactions.

## 2.07.2.2.1 Ketone nucleophiles with ketone electrophiles

Unmodified ketone is generally a less reactive electrophile; therefore, activated ketones are used in the intermolecular aldol reaction of ketone nucleophiles with ketone electrophiles. The crucial asymmetric tetrasubstituted carbon center of 107 is constructed with excellent stereoselectivity through the (S)-proline-catalyzed direct asymmetric aldol reaction between cyclohexanone 105 and ethyl phenylglyoxylate 106 under mild conditions carried out by the Maruoka group. The asymmetric synthesis of the carboxylic acid 108 as a key intermediate for the preparation of chiral (S)-oxybutynin (109), which is a prescribed muscarinic receptor antagonist, has been achieved (Scheme 17).<sup>51</sup>

Similarly, 1,2-diketones 111 are good electrophiles in aldol reaction, affording the corresponding 2-hydroxy 1,4-diketones 112 with high stereoselectivities reported by the Zhao group. This reaction provides an easy access to optically active tertiary alcohols 112 bearing two carbonyl groups for further transformation (equation 9).<sup>52</sup>

Scheme 17 Intermolecular aldol reaction of cyclohexanone with ethyl phenylglyoxylate.

Although chiral tertiary  $\alpha$ -hydroxy phosphonates 115 are potentially important chiral building blocks for the synthesis of bioactive compounds, there is no general method for their synthesis. The Zhao group demonstrated an (S)-proline-catalyzed cross-aldol reaction of acetone 113 with  $\alpha$ -keto phosphonates 114. Good yields and high enantiomeric purity up to 99% ee are observed (equation 10). <sup>53</sup> In addition, Hu and coworkers have presented the example of introducing primary amino acids into bispidine frameworks to generate catalysts that achieve highly enantioselective direct aldol reactions of  $\alpha$ -keto phosphonates and  $\alpha$ -ketoesters with acetone or cyclohexanone. <sup>54</sup>

The dynamic kinetic resolution of 2-oxo-3-arylsuccinates 116 has been achieved through (S)-proline-catalyzed aldol reaction of acetone 113 by the Zhang group, providing the desired adduct 117 in up to 72% yield with up to 99% ee (equation 11).<sup>55</sup>

The Gong group reported interesting study using (S)-proline derivatives 119 as an aldolase-type organocatalyst. Prolinamide 119, which is designed based on molecular recognition, catalyzes the aldol reaction of acetone 113 with aryl and alkyl  $\alpha$ -keto acids 118. The corresponding  $\gamma$ -keto- $\alpha$ -hydroxyl esters 121 with a tentary stereogenic center are obtained with excellent enantioselectivities. It is easy to separate the acidic aldols ( $\alpha$ -hydroxy carboxylic acids) and the basic catalyst 119 from the reaction mixture by an acid-base conversion strategy, and hence, recycling of the catalyst 119 is allowed (Scheme 18).

# 2.07.2.2.2 Ketone nucleophiles with aldehyde electrophiles

# 2.07.2.2.2.1 Catalyzed by secondary amine derivatives

The first intermolecular aldol reaction of a ketone with an aldehyde using (S)-proline was reported by the List and Barbas group in 2000 (Scheme 19). The intermolecular aldol reaction of acetone 113 with 4-nitrobenzaldehyde 122 ( $R^1 = 4 \cdot NO_2C_6H_4$ ) in dimethyl sulfoxide (DMSO) at room temperature for 4 h has furnished aldol 123a in 68% yield with 76% ee. The significant by-product is the only  $\alpha,\beta$ -unsaturated ketone formed through aldol or Mannich-type condensation. Aldol products 123 including aromatic as well as aliphatic groups are obtained, especially the reaction of acetone 113 with isobutyraldehyde 122 ( $R^1 = iPr$ ) gives aldol 123e in 97% yield and 96% ee. These findings have made outstanding progress in proline chemistry, because it had been

286

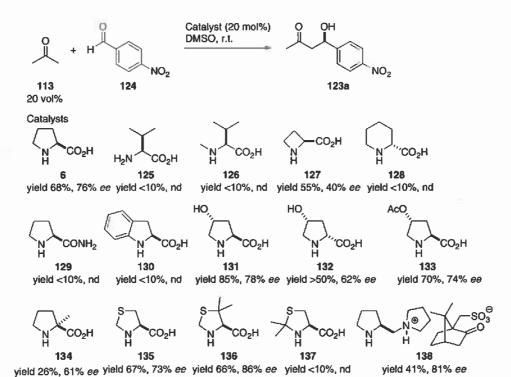
Scheme 18 Aldol reaction of acetone with anyl and alkyl  $\alpha$ -keto acids.

Scheme 19 First (S)-proline-catalyzed intermolecular aldol reaction.

known that proline reacts with an aldehyde to give several products such as the oxazolidinone, self-aldol product, and 1,3-dipolar cycloaddition product.

The five-membered pyrrolidine ring and the carboxylic acid moiety are crucial components for high reactivity and stereoselectivity (Scheme 20). <sup>57</sup> Simple natural amino acid such as (S)-valine 125 is a poor catalyst for the aldol reaction; in addition, N-methyl valine 126 is also ineffective. The size of the ring has significant effects on the reaction; thus, lower reactivities are shown with 2-azetidinecarboxylic acid 127 and pipecolic acid 128 than with (S)-proline. An acidic proton in appropriate spatial proximity is essential for efficient catalysis, because 2-pyrrolidine carboxamide 129 does not yield the desired aldol 123a. Substituents at the 4-position of proline scaffold 131–133 do not significantly affect the reactivity and stereoselectivity; however, substituent at the 2-position 134 provides aldol 123a in decreased yields. L-Thiaproline 135 shows approximately the same enantioselectivities as (S)-proline. Best enantioselectivity is achieved by 5,5-dimethylthiazolidine-4-carboxylic acid 136; in contrast, 2,2-dimethylthiazolidine-4-carboxylic acid 137 provides the aldol product 123a in dramatically decreased yields. Similarly, a diamine salt 138 containing ammonium ion as an acid moiety is an effective catalyst as well. Following these findings, aldol reactions using secondary amines, most of which make use of the pyrrolidine moiety, have been studied predominantly.

The initial screening of catalysts reported by Barbas for the asymmetric aldol reaction of acetone with 4-nitrobenzaldehyde illustrates the effect of a number of different modifications of the proline structure. However, there was still space for improvement, regarding yield, enantioselectivity, generality, and catalyst loading. Many catalysts have since been developed based on the structure of proline (Figure 4). For instance, 4-siloxyprolines 139,<sup>58-60</sup> prolinamides 140,<sup>61</sup> ethanolamine derivatives 141,<sup>62</sup> sulfonamide derivatives 142,<sup>63,64</sup> diamine catalyst 143,<sup>43,65,66</sup> tetrazole derivative 144,<sup>67-70</sup> and diarylprolinol 145<sup>71-74</sup> have been reported. Some common modifications to the proline structure involve increasing the hydrophobicity to improve its solubility in organic solvents and replacing the carboxylic acid with a variety of other hydrogen-bonding groups. Small peptides and their derivatives have also been evaluated as alternative catalysts for direct catalytic asymmetric aldol reactions.<sup>75-77</sup>



Scheme 20 Catalyst screening in intermolecular aldol reaction.

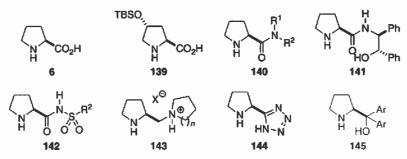


Figure 4 Organocatalysts derived from proline or hydroxyproline.

Secondary amine catalysts having pyrrolidine units that are not derived from proline exist in the literature (Scheme 21). The  $C_2$ -symmetric (2S,5S)-pyrrolidine-2,5-dicarboxylic acid (147, 30 mol%) has been used as a combination with triethylamine (30 mol%) in the intermolecular aldol reaction to afford the aldol product 146 in good yields and moderate enantioselectivities.<sup>78</sup> Phosphinyl oxide pyrrolidine 148 has also been reported to afford the desired aldol adduct 146 in 90% yield and in 85% ee.<sup>79</sup> Moderate yields have been obtained in aldol reactions catalyzed by (S,S,S)-perhydroindolic acid 149, with the best enantioselectivities being obtained for aromatic aldehydes possessing electron-withdrawing groups.<sup>80</sup>

Secondary amine catalysts that do not contain pytrolidine units also exist in the literature. Maruoka and coworkers have found that the binaphthyl-based amino acid 151 is an efficient catalyst for direct asymmetric aldol reaction of acetone 113 with the aldehydes (Scheme 22). With 5 mol% of catalyst 151, adduct 123a was obtained in good yield and enantioselectivity (82%, 95% ee). The same reaction with (S)-proline 6 gave the desired aldol product 123a in low yield with moderate enantioselectivity, as well as a side product, 1,3-oxazolidine 150 (48% yield based on proline), which is derived from (S)-proline itself and two equivalents of 4-nitrobenzaldehyde 124. However, the formation of such a by-product was not observed with catalyst 151 because of its structural stability. They also designed a biphenyl-based amino acid of type 152, which is highly substituted with electron-donating methoxy groups, with the expectation of the increasing nucleophilicity of the amino moiety. In the case of catalyst 152, the catalyst loading could be decreased to only 0.1 mol% in acetone without loss of yield or enantioselectivity. 83

It is always required to allow for the formation of self-aldol products as a by-product, when  $\alpha$ -unsubstituted aldehydes 153 are used as a substrate. The List group has demonstrated the (S)-proline-catalyzed cross-aldol reaction of acetone 113 with

288

yield 65%, 26% de, 89% ee yield 55%, 34% de, 87% ee yield 90%, 38% de, 85% ee

yield 50%, 78% ee

Scheme 21 Pyrrolidine-based catalysts for aldol reaction of cyclohexanone.

Scheme 22 Direct asymmetric aldol reaction catalyzed by (S)-proline vs. axially chiral catalysts.

 $\alpha$ -unsubstituted aldehyde 153, in which aldol products 154 are obtained with moderate enantioselectivities along with the formation of comparable amounts of  $\alpha,\beta$ -unsaturated ketones 155 (Scheme 23). A concise new synthesis of (S)-ipsenol (156) is developed using this strategy.

Scheme 23 Cross-aldol reaction of acetone with a-unsubstituted aldehydes.

Aldol reactions of  $\alpha$ -hydroxyketone 157 with an aldehyde 122 create chiral *anti*-1,2-diols 158, which are common structural motifs found in a vast array of natural and biologically active molecules. The List group reported highly diastereo- and enantioselective synthesis of the *anti*-1,2-diol 158 (Scheme 24, see also Scheme 36).

**Scheme 24** Anti-selective aldol reactions of  $\alpha$ -hydroxyacetone with aldehyde.

The following models account for the above *anti*-stereoselectivities (Scheme 25). (E)-Enamine intermediate 160 predominates because of steric interactions in (Z)-enamine 159 in (S)-proline-catalyzed reaction, thus a carbon–carbon bond is formed between the si-face of the (E)-enamine 160 and the re-face of aldehyde 122 to give the *anti*-isomer 158 (see also Scheme 37).

Scheme 25 Proposed transition state model for anti-selective aldol reaction.

A biomimetic asymmetric synthesis of carbohydrates including aminosugars has been accomplished by a proline-catalyzed aldol reaction of the protected dihydroxyacetone 162 with aldehydes 122 reported independently by the Enders  $^{86}$  and the Barbas  $^{87}$  group (Scheme 26, see also Schemes 38 and 39). (S)-proline-catalyzed aldol reaction between dihydroxyacetone equivalent 162 with aldehyde 122 gives the corresponding anti-polyols 163a-d in good yields with > 90% ee. This new organocatalytic  $C_3+C_n$  strategy leads directly to anti-selectively protected simple sugars and amino sugars.

Scheme 26 Anti-selective aldol reactions of protected dihydroxyacetone with aldehyde.

The Kotsuki group has investigated the aldol reaction under unconventional reaction condition, that is, under high pressure (equation 12). Somparable enantioselectivities are observed; however, aldol 123c is obtained in an improved yield at 0.2 GPa. In addition, this process does not require the use of DMSO as a cosolvent.

Recently, ionic liquids have attracted great interest in organic synthesis, as their properties such as nonvolatility and insolubility in some solvents are suitable for environmentally friendly synthesis. (S)-proline in imidazolium-based ionic liquids has been successfully used as an efficient catalyst for direct aldol reactions carried out by the Loh group (Scheme 27, see also Scheme 89). Comparable or better enantioselectivities are observed compared with that in conventional organic solvents; in addition, less or no elimination product is detected.

Scheme 27 Aldol reactions in ionic liquids.

Chiral solvents should have an effect on stereoselectivity. Recently, the North group reported (S)-proline-catalyzed aldol reaction in chiral propylene carbonate 166 as a solvent (Scheme 28). When enantiomerically pure propylene carbonate is used, the combination of (R)-proline 6 and (R)-carbonate 166 constitutes a matched pair, whereas (S)-proline 6 and (R)-carbonate 166 constitutes a mismatched pair.

Scheme 28 (S)-proline-catalyzed aldol reactions in chiral solvent.

The Walsh group reported interesting dynamic kinetic resolution of racemic naphthamide 168 (equation 13).<sup>91</sup> Excellent stereoselectivities and good yield are observed, wherein the stereochemistry of the atropisomeric amide 169 chiral axis and a stereogenic center are simultaneously controlled.

Catalysts are traditionally designed and optimized to mediate a single reaction; however, (S)-proline is capable of catalyzing multiple reactions in a one-pot reaction. Direct asymmetric assembly of acetone 113, aldehyde 170, and azodicarboxylic acid ester 171 provides an optically active  $\beta$ -amino alcohol 173 in good yields with excellent enantiomeric excess carried out by the Barbas group. (Scheme 29). This reaction involves stepwise reaction sequence through  $\alpha$ -amination of the aldehyde 170 with the electrophilic azo compound 171 followed by aldol reaction of acetone 113 with the aldehyde intermediate 172. Both aldehyde and ketone as nucleophiles are used in one-pot operation.

Scheme 29 Assembly reactions of acetone and dibenzyl azodicarboxylate with aldehyde.

After List-Barbas aldol reaction reported in 2000, intermolecular aldol reaction is recently employed for the synthesis of biologically active complex molecules. Epothilones A (179) and B (180) are naturally occurring 16-membered macrolides, which are produced by the myxobacterium *Sorangium cellulosum*. Although no structural similarities between epothilones and taxol 62 are observed, they exhibit similar biological activities in vitro. Key structure 177 of epothilones is constructed through intermolecular and intramolecular aldol reactions carried out by the Avery group (Scheme 30). 93 The δ-ketoaldol 175 is synthesized in 75% yield with >99% ee by the (R)-proline-catalyzed aldol reaction of acetone 113 with a pivaldehyde-like aldehyde 174. Pyrrolidine-catalyzed intramolecular aldol reaction of δ-ketoaldol 175 furnishes enone 176 in 76% yield without protection of the hydroxy group. Further oxidation of the silyl-protected enone leads to the key product 177, and then epothilones 179 and 180 could be produced according to the known procedure.

Scheme 30 Epothilone synthesis through intermolecular aldol reaction.

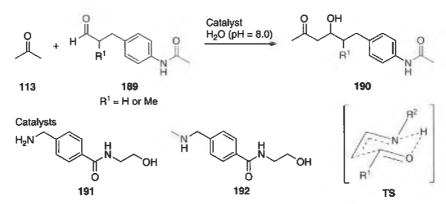
Apratoxin A (188), isolated from the marine cyanobacterium *Lyngbya majuscula* Harvey ex Gomont, possesses IC<sub>50</sub> values for *in vitro* cytotoxicity against human tumor cell lines. Interestingly, this cyclodepsipeptide of mixed peptide–polyketide biogenesis bears a thiazoline ring flanked by polyketide portions including an unusual methylation pattern. The Doi and Takahashi group have employed the (*R*)-proline-catalyzed intermolecular aldol reaction of acetone 113 with pivaldehyde 181 in multigram scale to synthesize the polyketide building block 182 (Scheme 31). <sup>94,95</sup> The coupling of polyketide 186 with peptide 187, followed by macrolactamization between the proline and *N*-methylisoleucine residues provides apratoxin A (188).

Scheme 31 Apratoxin A synthesis through proline-catalyzed aldol reaction.

## 2.07.2.2.2.2 Catalyzed by primary amine derivatives

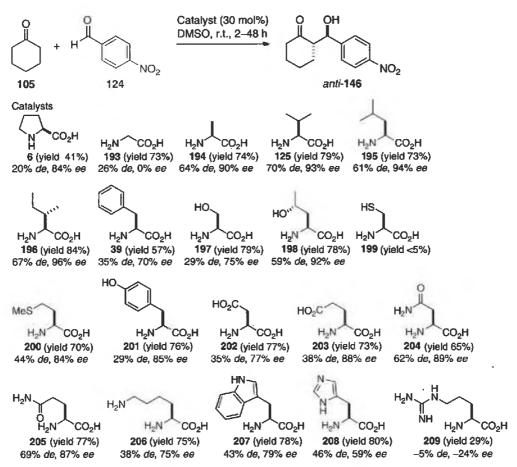
In the seminal paper by List, Barbas, and Lerner, 2,57 it has been reported that (S)-proline 6 promotes the enantioselective aldol reaction of acetone 113 and 4-nitrobenzaldehyde in DMSO, whereas primary amino acids such as histidine, valine, threonine, and phenylalanine do not (see Scheme 20). However, a few years earlier, in 1995, Reymond and coworkers found that intermolecular aldol reactions of acetone 113 with aldehydes 189 could be catalyzed by both primary and secondary amines. They also studied a variety of amine-catalyzed aldol reactions in water and reported that primary amines react faster than secondary amines, with the exception of proline. Primary amine 191 was found to catalyze the stereoselective aldol reaction of acetone 113 to aldehyde 189 10 times faster than secondary amine 192 (Scheme 32). For this reaction, Houk suggested that primary enamine-mediated aldol reactions involve half-chair transition states TS with hydrogen bonding leading to proton transfer (Scheme 32). This leads to charge stabilization and low activation energies as compared with secondary enamine-mediated aldol reactions. Ever since these findings, the search for efficient organocatalysts has been extended to the family of primary amines. In this section, intermolecular aldol reactions using primary amines are reviewed. (18–101)

There have been reports that primary amino acids can promote aldol reaction, and in some cases, excellent enantioselectivities have been achieved. For instance, Córdova and coworkers have examined the aldol reaction of cyclohexanone 105 and 4-nitrobenzaldehyde 124 using 13 of the 20 natural amino acids under aqueous conditions. <sup>102</sup> A report by Hayashi and coworkers has shown a systematic study of all 20 proteinogenic amino acids for the aldol reaction of 105 and 124 in DMSO (Scheme 33). <sup>103</sup> The reaction proceeds with all amino acids except cysteine 199, and aldol products 146 are obtained enantioselectively except with glycine 193. The obtained yields, diastereoselectivities, and enantioselectivities depended on the amino acid tested. Even glycine 193, <sup>104</sup> the simplest amino acid, allows the aldol reaction to afford the desired product 146 in good yield. Moreover, even alanine 194, the simplest chiral amino acid, furnishes the aldol product 146 with good enantioselectivity. Perhaps surprisingly, proline 6 is not the amino acid that delivers the best results; many other amino acids, all primary amines, render higher yields and enantioselectivities. In addition, Lu and coworkers have reported 12 of the 20 proteinogenic amino acids for the aldol reaction of



Scheme 32 Intermolecular aldol reactions catalyzed by primary amines and half-chair transition state model.

105 and 124 in H<sub>2</sub>O, <sup>105</sup> for which they find tryptophan 207 to be the best catalyst. After screening solvents, water is found to be the best solvent for tryptophan catalyst.



Scheme 33 Systematic studies of all 20 proteinogenic amino acids in aldol reactions.

Córdova and Himo carried out computational studies to understand the observed reactivity of amino acid-catalyzed reactions. Density functional theory calculations were performed on alanine-catalyzed aldol reactions to provide a key understanding of the reaction mechanism. The carboxylic acid-associated enamine mechanism 210 is more favorable (Figure 5). The aminemediated enamine mechanism 211 and the enaminium-mediated mechanism 212 are less favorable, as much higher activation energies are required.

Figure 5 Possible transition state models of (S)-alanine-catalyzed aldol reaction.

Maruoka and coworkers reported intermolecular aldol reactions that resulted in both enantiomeric forms of the aldol product 146 by using two different chiral organocatalysts 214 and 215 (Scheme 34). 107 These catalysts can be synthesized from a common precursor 213 in a three-step sequence. The asymmetric aldol reaction of cyclohexanone 105 and 4-nitrobenzaldehyde 124 catalyzed by 214 or 215 in THF-H<sub>2</sub>O afforded aldol products 146 in excellent yield, anti-selectivity, and excellent enantioselectivity, but with opposing absolute configurations. After elucidating the optimal conditions for the aldol reaction, the researchers studied the scope of aldol reactions using various cyclic ketones and substituted aryldehydes. Both catalysts generally exhibited high anti-selectivity and excellent enantioselectivity. In the case of cyclohexanone substrates, the catalyst loading could even be reduced to 1 mol% without compromising the high anti-selectivity and enantioselectivity.

Scheme 34 Asymmetric aldol reactions using two different chiral organocatalysts synthesized from common chiral source.

Formaldehyde 217 is one of the most important  $C_1$  units as electrophiles in organic synthesis; in addition, aqueous formaldehyde solution, that is, formalin, is a very cheap chemical. Water-compatible Lewis acids have developed the use of aqueous formaldehyde solutions in the Mukaiyama aldol reaction. Although (S)-proline-catalyzed direct  $\alpha$ -hydroxymethylation of cyclohexanone with aqueous formaldehyde solution furnishes the corresponding  $\alpha$ -hydroxymethylated aldol 218b with excellent enantioselectivity, (S)-proline is not an effective catalyst for aldol reaction of five-, seven-, and eight-membered cyclic ketones 216. Recently, the Mase and Takabe group has developed enantioselective  $\alpha$ -hydroxymethylation of these cyclic ketones 216 using simple amino acids such as (S)-threonine 198. In (S)-proline catalysis highly strained cyclic iminium or enamine intermediates would be formed; in contrast, (S)-threonine catalyst forms a more flexible intermediate, but weakly fixed by intramolecular hydrogen bonding, to react with formaldehyde through the proposed transition state TS (Scheme 35).

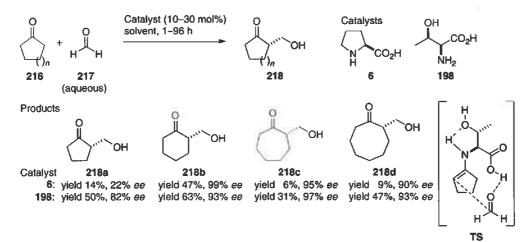
# 2.07.2.2.2.3 Syn-selective aldol reactions

Chiral 1,2-diols are common structural motifs found in biologically active molecules. Efficient enantioselective syntheses of anti1,2-diols 158 have been developed using enamine-based organocatalytic strategies with most research occurring from 2000 to
2006 as noted in Scheme 24. Before 2007, there were no reports on the synthesis of syn-1,2-diols 158 through an organocatalytic
approach; however, use of primary amine-containing amino acid 219 derived from commercially available (S)-threonine overcame this synthetically important challenge. An aldol reaction catalyzed by 219 with the unmodified  $\alpha$ -hydroxyketone 157
furnishes highly diastereomerically as well as enantiomerically enriched syn-1,2-diol 158 in excellent yield (Scheme 36).

Introduction of not only *tert*-butyl group but also siloxy groups at the hydroxyl function of (S)-threonine by Lu and coworkers resulted in efficient hydrophobic organocatalysts for this type of syn-selective reaction.

When (S)-proline 6 is used as a catalyst, the (E)-enamine intermediate 160 predominates due to steric interactions in (Z)-enamine 159 (Scheme 25). In contrast, with primary amine-containing amino acid 219, (Z)-enamine 221 of a-hydroxyketone 157 predominates over (E)-enamine 220 due to intramolecular hydrogen bonding, and thus, syn-diastereoselectivity has been observed through the bond-forming transition state 222 (Scheme 37).

Furthermore, the (S)-threonine-based primary amine 219 is a powerful organocatalyst that has been used to synthesize L-rhamnulose and p-fructose derivatives 225 from protected dihydroxyacetone 223 as a donor. This new strategy offers the



Scheme 35  $\omega$ -Hydroxymethylation of cycloalkanones with aqueous formaldehyde solution.

Scheme 36 Syn-selective aldol reactions of hydroxyacetone with aldehyde.

Scheme 37 Predicted transition state model for syn-selective aldol reactions.

organocatalytic mimics of the enzymes 1-rhamnulose 1-phosphate aldolase and p-fructose 1,6-diphosphate aldolase (Scheme 38). 113,114

Carbohydrates play diverse and essential roles in biology, medicine, and industry and thus synthesizing them stereoselectively is an important concern in organic chemistry, and specifically in aldol chemistry. Therefore, asymmetric aldol reactions between the unprotected dihydroxyacetone 226 and aldehydes 122 have been a challenging topic in organocatalysis. For example, no enantioselection was observed in the (S)-proline or (S)-1-(pyrrolidin-2-ylmethyl)pyrrolidine-catalyzed aldol reaction of dihydroxyacetone 226 with aldehyde 122 reported by the Barbas group in 2002, though this reaction interestingly proceeded in aqueous conditions.<sup>115</sup> In 2007, the same group overcame this difficulty by the use of (S)-threonine-based primary amine 219

296

Scheme 38 Syn-selective aldol reactions of protected dihydroxyacetone with aldehyde.

(Scheme 39). 113 DMF and 5-methyl-1H-tetrazole are identified as an optimal solvent and acid additive combination, in which syn-aldol products 227 are obtained in good yield with >92% ee.

Scheme 39 Syn-selective aldol reaction of unprotected dihydroxyacetone with aldehyde.

Aldol reactions of linear aliphatic ketones 1 with aldehydes 228 are synthetically important; however, regioselectivity and stereoselectivity are difficult to control. The chiral primary-tertiary diamine 229, derived from nonamino acids, in combination with trifluoromethanesulfonic acid (TfOH) catalyzes the aldol reaction with high regio-, syn-diastereo-, and enantioselectivity (Scheme 40). 116 The reaction of 2-butanone occurred preferentially at the methylene carbon with good regioselectivity, favoring the branched aldol product 230a in high enantioselectivity. More interestingly, the branched product 230a was obtained with unexpected diastereoselectivity, favoring the syn-isomer; in contrast, the reaction with (S)-proline derivatives generated anti-selective products with acyclic ketones. In the case of cyclic ketone as donors, anti-product 146 was obtained.

The (Z)-enamine transition state TS-1 was proposed in the sym-selective reaction of an acyclic ketone donor with an aldehyde acceptor catalyzed by 229. In this model, the protonated tertiary amine serves as a hydrogen-bonding donor. The formation of the (E)-enamine of the acyclic ketone is disfavored due to steric repulsion between  $R^1$  and  $R^2$  substituents. The reaction of cyclohexanone, which is only capable of forming (E)-enamine transition state model TS-2, gave *anti*-diastereoselectivity (Figure 6).

#### 2.07.2.2.3 Aldehyde nucleophiles with ketone electrophiles

Controlling the chemo- and stereoselectivities in intermolecular aldol reaction using aldehyde nucleophiles is one of the most challenging topics in organic synthesis. The direct catalytic asymmetric cross-aldol reaction using a nucleophilic aldehyde with a

Scheme 40 Aldol reactions catalyzed by chiral primary-tertiary diamine.

Figure 8 Predicted transition state models for aldol reactions catalyzed by chiral primary-tertiary diamine.

ketone electrophile has been reported by the Jørgensen group (Scheme 41).  $^{117}$   $\alpha$ -Unsubstituted aldehydes 153 are used in (S)-proline-catalyzed cross-aldol reaction with activated ketomalonates 231. The desired products 232 are obtained in excellent yields and enantioselectivities, though a substoichiometric amount of catalyst is required. The asymmetric intermolecular aldol reaction of aldehyde with unactivated simple ketone is still difficult to achieve (see Section 2.07.2.1.3).

Scheme 41 Aldol reactions of aldehyde nucleophile with activated ketone electrophile.

## 2.07.2.2.4 Aldehyde nucleophiles with aldehyde electrophiles

# 2.07.2.2.4.1 Aldol reactions of aldehyde nucleophiles

It has been a chemist's dream to accomplish the highly controlled asymmetric cross-aldol reaction of a wide variety of abundant aldehydes. Although many transformations of aldehyde functional group are well known, aldehydes apparently have a tendency to polymerize under acidic or metal-catalyzed conditions. The first (S)-proline-catalyzed direct self-aldol reaction of acetaldehyde 233 was disclosed by the Barbas group (Scheme 42).<sup>118</sup> (5S)-Hydroxy-(2E)-hexenal 235 with 90% ee in a low chemical yield is obtained through the self-aldol followed by Mannich-type condensation.

Scheme 42 Self-aldol reaction of acetaldehyde.

The Barbas group reported the directed asymmetric assembly of simple three aldehyde molecules into stereochemically complex triketides 236 (Scheme 43). 119 The pyranose products 236 including four asymmetric centers are constructed by (S)-proline-catalyzed double aldol reactions. Despite modest stereoselectivities, this approach may warrant consideration as a prebiotic route to sugars and polyketides.

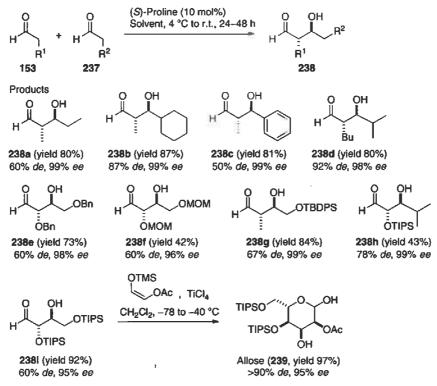
**Scheme 43** (S)-proline-catalyzed assembly of aldehydes to synthesize pyranoses.

Nonequivalent aldehydes must selectively partition into two discrete components, that is, a nucleophile and an electrophile, in cross-aldol reaction of  $\alpha$ -unsubstituted aldehydes. The MacMillan group has achieved this challenging aldol reaction, and syringe addition of electrophilic aldehyde is a key to high reaction efficiency (Scheme 44, products 238a-238d). <sup>120</sup> By slowly adding the donor aldehyde 153 to 10 equivalents of the acceptor aldehyde 237, the desired cross-aldol product 238 was obtained in good yield and enantioselectivity with only 10 mol% (S)-proline. This excellent procedure is employed in producing protected erythrose using  $\alpha$ -oxyaldehydes (Scheme 44, products 238e-238i). <sup>121</sup> Furthermore, a synthetic route based on aldol coupling of three aldehydes is accomplished for the *de-novo* synthesis of hexoses in only two steps, that is, the (S)-proline-catalyzed self-aldol reaction of  $\alpha$ -oxyaldehydes, followed by a Lewis acid-catalyzed Mukaiyama aldol addition-cyclization afforded the protected allose 239 (Scheme 44). <sup>122</sup> This strategic synthetic procedure is very helpful to prepare differentially protected glucose, allose, and mannose stereoisomers in high yields and stereoselectivities simply by changing the solvent and the Lewis acid used.

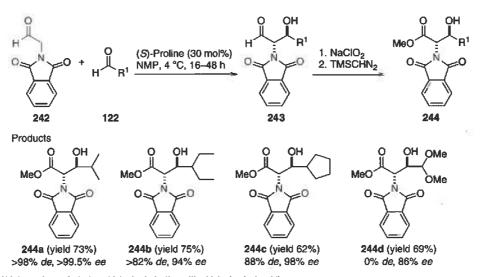
One-pot de-novo synthesis of carbohydrates has also been accomplished by (S)-proline-catalyzed trimerization of  $\alpha$ -benzyloxyaldehyde 240 carried out by the Córdova group (equation 14). Let  $\alpha$  Chemical yield is moderate, but absolute stereocontrol of the protected allose 241 is achieved by this direct  $C_2+C_2$  methodology in one-pot operation.

Not only  $\alpha$ -oxyaldehydes but also  $\alpha$ -aminoaldehydes 242 are also investigated in direct aldol reactions. The Barbas group reported that  $\beta$ -hydroxy- $\alpha$ -amino acid derivatives 244 were prepared by the (S)-proline-catalyzed aldol reaction of glycine aldehyde 242 with aldehyde electrophiles 122 (Scheme 45). The reactions afford anti- $\beta$ -hydroxy- $\alpha$ -amino aldehydes 243 in good yields with high diastereo- and enantioselectivities, which are easily transformed into  $\beta$ -hydroxy- $\alpha$ -amino acid derivatives 244.

Although proline itself can catalyze cross-aldol reactions between different aldehydes in excellent enantioselectivities and high yields, some problems currently exist: (1) preventing self-aldol reactions without recourse to slow addition techniques and (2) development of syn-selective aldol reactions, as anti-products usually predominate. MacMillan and coworkers have reported an organocatalytic self-aldol reaction of propanal 170 using imidazolidinone catalysts 254 (Scheme 46), 125 and under these reaction conditions, the initial aldol dimerization adduct 246 undergoes rapid transformation into hemiacetal 247. This 'self-termination'



Scheme 44 (S)-proline-catalyzed cross-aldol reaction of  $\alpha$ -unsubstituted aldehydes.



Scheme 45 Aldol reactions of glycine aldehyde derivative with aldehyde electrophile.

step fortuitously protects the product from participating in further aldol processes. Methanolysis of this aldol hemiacetal product 246 in situ then allows direct access to a stable dimethoxyacetal 247 without loss in enantiopurity or diastereoselectivity.

Addition of  $\alpha$ -unsubstituted aldehyde 153 by means of a syringe pump to a variety of aldehyde acceptors 224 effectively prevents homodimerization while giving the desired cross-aldol product 248 in good yield and excellent enantioselectivity (Scheme 47). The resulting syn/anti-selectivity was almost the same as with proline, but the opposite enantiomer was obtained.

The high selectivity of this reaction is thought to arise from the selective formation of the (E)-iminium isomer during the transition state to avoid nonbonding interactions with the bulky *tert*-butyl group, as well as the prevention of the *re* face of the enamine 249 from participating in carbonyl addition due to the protruding benzyl group from the catalyst framework (Figure 7).

Scheme 46 Imidazolidinone-catalyzed self-aldol reaction.

Scheme 47 Imidazolidinone-catalyzed cross-aldol reaction.

In contrast, when (S)-proline is used as a catalyst, the aldehyde acceptor is activated by coordination to proline's carboxylic acid moiety 250.

Figure 7 Proposed intermediates in the aldol reaction comparing MacMillan's catalyst to (S)-proline.

An axially chiral binaphthylsulfonamide derivative 251 has been successfully used as the catalyst in a cross-aldol reaction between aliphatic 153 and aromatic aldehydes 224 as well as ethyl glyoxylate or phenylglyoxal, affording syn-isomers 252 with excellent enantiomeric excess (Scheme 48).<sup>126</sup>

A chemo- and stereoselective direct cross-aldol reaction between aliphatic aldehydes 253 and  $\alpha$ -chloroaldehydes 254 has been succeeded as a method for the formation of the cross-aldol adduct 257 with both diastereo- and enantiocontrol; in addition, either anti- or syn-aldol adducts 257 were obtained in good to excellent stereoselectivities by use of (S)-proline or the axially chiral amino sulfonamide 255 as the catalyst (Scheme 49). 127

The *syn*-selectivity in the binaphthylsulfonamide-catalyzed aldol reaction is believed to proceed as follows: It would be difficult for *anti*-enamine TS-1, generated from a donor aldehyde and the catalyst, to react with an acceptor aldehyde that is activated by the distal acidic proton of the sulfonamide catalyst. The cross-aldol product would thus be expected to proceed through the more favorable *syn*-enamine TS-2 (Scheme 50). 126

Scheme 48 Binaphthylsulfonamide-catalyzed syn-selective cross-aldol reaction.

Scheme 49 Binaphthylsulfonamide-catalyzed syn-selective cross-aldol reaction of 2-chloroaldehyde.

Scheme 50 Predicted transition state models for cross-aldol reaction catalyzed by binaphthylsulfonamide.

Although impressive, the synthetic scope of (S)-proline is not sufficient to address all aspects of the direct aldol reaction. For example, the synthesis of aldols with quaternary carbon atoms is one of the most challenging topics in asymmetric organic chemistry that is not addressed efficiently with (S)-proline catalysis, that is, aldol 259a is obtained in 34% chemical yield with 80% ee in the presence of (S)-proline (30 mol%) after 72 h stirring. Enamine formation is not effective in this condition, thus

1,3-dipolar cycloaddition product 260 through decarboxylation of iminium intermediate derived from (S)-proline and aldehyde 224 is formed. The Tanaka and Barbas group identified chiral-amine/acid combinations 96 as a bifunctional catalyst through a fluorescence-based evaluation system for asymmetric direct catalytic aldol reactions of  $\alpha$ , $\alpha$ -dialkyl aldehydes 258 with aryl aldehydes 224 (Scheme 51). 66 Aldols 259 bearing quaternary carbon atoms are obtained with excellent enantioselectivities.

Scheme 51 Synthesis of aldols with quaternary carbon centers catalyzed by a chiral amine/acid combination catalyst.

Trifluoromethyl-substituted diarylprolinol 262, which is easily prepared from (S)-proline, is an efficient catalyst in several aldol reactions, in which proline does not afford the good results. Ethyl glyoxylate is a useful electrophile, which is commercially available as its polymer form 261 in toluene solution. Usually, its monomer, prepared through pyrolysis just before use from the polymer, is used in aldol reaction, and it is a synthetic advantage if its polymer can be directly employed in the reaction. The cross-aldol reaction of ethyl glyoxylate using its polymer solution can be catalyzed by diarylprolinol 262 to afford the aldol product with excellent diasereo- and enantioselectivity (Scheme 52).<sup>74</sup>

Scheme 52 Diarylprolinol-catalyzed cross-aldol reaction of aldehyde with ethyl glyoxylate using its polymer solution.

This catalyst 262 is also effective in the aldol reaction of chloroacetaldehyde hydrate 265 (Scheme 53)<sup>128</sup> and pyruvaldehyde hydrate 268 (Scheme 54),<sup>129</sup> both of which are commercially available as an aqueous solution. In the reaction of chloroacetaldehyde, aldol product 266 was treated with Wittig reagent, followed by the addition of base, which gave epoxy ester 267 with excellent enantioselectivity in one-pot operation.

Scheme 53 Diarylprolinol-catalyzed cross-aldol reaction of aldehyde with chloroacetaldehyde.

Scheme 54 Diarylprolinol-catalyzed cross-aldol reaction of aldehyde with pyruvaldehyde.

Trifluoromethylacetaldehyde ethyl hemiacetal 270 can also be employed as an electrophilic aldehyde equivalent. Acetal 270 was used directly in the aldol reaction catalyzed by trifluoromethyl-substituted diarylprolinol 262 to afford the aldol product 271 with excellent enantioselectivity (Scheme 55). 130

An impressive synthesis of natural product using organocatalytic methodology including a proline-catalyzed  $\alpha$ -aminoxylation, Michael addition, and aldol reaction is accomplished by the MacMillan group (Scheme 56). Littoralisone (279) has a six-membered acetal, an adjacent nine- and five-membered lactones, cyclobutane and cyclopentane rings, and saccharide; in addition, 14 stereocenters including 6 contiguous stereocenters are found. Iridolactone 277 and 2-cinnamoyl saccharide 278 are key

304

Scheme 55 Diarylprolinol-catalyzed cross-aldol reaction of aldehyde with trifluoromethylacetaldehyde ethyl hemiacetal.

intermediates according to their synthetic strategy. (S)-Proline-catalyzed  $\alpha$ -aminoxylation of aldehyde 273 derived from readily available (S)-citronellol 272 with nitrosobenzene furnishes the corresponding oxyaminoaldehyde. Subsequently, Horner-Wadsworth-Emmons olefination and cleavage of the aminoxy bond afforded the  $\alpha,\beta$ -unsaturated ester 274 in a single operation from 273. Intramolecular Michael reaction of enal 275 with (S)-proline as a catalyst gives the bicyclic product 276, then further transformation leads the key iridolactone 277. Another key 2-cinnamoyl saccharide 278 is prepared by (R)-proline-catalyzed dimerization of benzyloxyacetaldehyde 240, followed by Mukaiyama aldol reaction (see Scheme 44). Synthesis of the target littoralisone (279) is accomplished by the glycosidic union of 277 and 278, subsequently intramolecular [2+2] photocyloaddition.

Scheme 56 Littoralisone synthesis through proline-catalyzed a-aminoxylation, Michael addition, and aldol reaction.

Another application of organocatalyst-mediated aldol reaction to total synthesis of natural product is the synthesis of (+)-cytotrienin A (288). Cytotrienin A is a microbial antitumor secondary metabolite, isolated from a fermentation broth of Streptomyces sp. RK95-74 from soil. 132 It possesses a (E,E,E)-triene within a 21-membered cyclic lactam, which also contains four-chiral centers, common structural features of the ansamycin class of natural products. The three contiguous chiral centers have been constructed by the asymmetric aldol reaction as a key step, in which surfactant-proline conjugated catalyst 281 is effective to afford the aldol product 282 with good diastereoselectivity and excellent enantioselectivity in contrast to the low diastereoselectivity in the case of proline-mediated reaction. The chirality at C-3 is controlled by proline-catalyzed a-aminoxylation of aldehyde 284. Ring-closing metathesis of diene 287 and diene moieties to construct triene moiety afforded the 21-membered macrolactam 288 (Scheme 57).

Scheme 57 Asymmetric total synthesis of (+)-cytotrienin A.

### 2.07.2.2.4.2 Aldol reactions of acetaldehyde nucleophile

When acetaldehyde 233, a simple aldehyde that is unsubstituted at the  $\alpha$ -position, is employed in an aldol reaction, it is expected to act as both a reactive electrophile and a nucleophile. The aldol product itself is an unhindered nucleophile, and thus suppression of further reaction is a difficult problem. In fact, Barbas and coworkers reported that 5-hydroxy-2-hexenal 235, a trimerization product of acetaldehyde 233, was obtained in 10% yield and 90% ee when acetaldehyde 233 was treated with (S)-proline (Scheme 42). In contrast, Hayashi and coworkers developed a diarylprolinol catalyst 262 that allowed acetaldehyde dimerization (Scheme 58). The aldol product that was generated reacted immediately with another acetaldehyde molecule

through the oxygen atom to generate a cyclic hemiacetal 289, which suppressed the undesirable overreaction; side reactions, such as dehydration to afford 2-butenal, were not observed.

Scheme 58 Self-aldol reaction of acetaldehyde using diarylprolinol.

When diarylprolinol 262 was employed in the aldol reaction of acetaldehyde 233 with 2-chlorobenzaldehyde 291, the crossaldol product 292 was generated in good yield with nearly complete enantioselectivity (Scheme 59–1).<sup>71</sup> The reaction proceeded efficiently with electron-deficient aromatic aldehydes and olefinic aldehydes. Hayashi and coworkers have also developed the asymmetric aldol reaction of acetaldehyde 233 with isatin derivatives 293, catalyzed by 4-hydroxydiarylprolinol 294, to afford the desired aldol product 295 in high enantioselectivity (Scheme 59–2).<sup>73</sup> They applied the present method to the short syntheses of ent-convolutamydine E and CPC-1, as well as to a route toward madindolines A and B.

Scheme 59 Diarylprolinol-catalyzed cross-aldol reactions using acetaldehyde as a nucleophile.

The reaction shown in Scheme 59 is thought to proceed as follows: The diarylprolinol catalyst 262 reacts with acetaldehyde 233 to generate the corresponding *anti*-enamine 296, which then reacts with an electrophilic aldehyde through transition state 297 (Figure 8). The aldehyde is thus activated by coordination to the proton of the hydroxyl group through a hydrogen bond.

## 2.07.2.3 Aldol Reactions in Water, in the Presence of Water, on Water, and by Water

Reactions in water, in the presence of water, on water, and by water have attracted a great deal of attention because water is an environmentally friendly and safe medium, which avoids the problems of pollution that are inherent to organic solvents. [1,3-1,35] Aldol reactions using organocatalysts in the presence of water are not only 'green', but water can also improve reactivity and selectivity in some cases. Water has been known to exhibit special properties when compared with common organic solvents; for

Figure 8 Enamine intermediate and the transition state model.

instance, Breslow and coworkers reported a rate acceleration of the Diels-Alder reaction 'in water,' wherein the reaction was performed at very high dilution to dissolve all the reactants. 136,137 In contrast, Sharpless and coworkers described 'on water' reaction conditions under which substantial rate acceleration was observed when the organic reactants were insoluble in the aqueous phase. 138 In this section, aldol reactions in water, in the presence of water, on water, and by water using secondary or primary amines are reviewed. 139-142

## 2.07.2.3.1 Water as an additive

Water in organocatalytic intermolecular aldol reactions has been investigated by the Barbas group, ever since their seminal full paper in 2001.<sup>57</sup> Therein, it was reported that the reaction of acetone 113 and 4-nitrobenzaldehyde 124 tolerated a small amount of water (<4% v/v) without affecting the enantiomeric excess of the aldol product 123a. However, increasing the amount of water decreased the enantioselectivity and the rate of formation of the aldol product 123a. Yamamoto and coworkers subsequently discovered a proline-derived tetrazole species that affects aldol catalysis in the presence of at least 100 mol% water.<sup>68</sup> In their study, only hydrated aldehydes were found to be reactive. Pihko also demonstrated that water has an accelerating effect on proline-catalyzed ketone-aldehyde aldol reactions.<sup>143</sup> Water can also accelerate aldol reactions catalyzed by nonproline amino acids. Amedjkouh reported that valine 125 was able to catalyze the aldol reaction between acetone 113 and 4-nitrobenzaldehyde 124 in good stereoselectivity when employed in aqueous DMSO (equation 15).<sup>144</sup> In contrast, no reaction was observed when reacting in the presence of 20 mol% of 113 in pure DMSO. In an aqueous DMSO reaction medium, good results were also obtained with other amino acids, such as phenylalanine and aspartic acid.

Córdova and coworkers examined the aldol reaction of cyclohexanone 105 and 4-nitrobenzaldehyde 124 using 13 of 20 proteinogenic amino acids and discovered that excellent enantioselectivity was obtained when either valine or isoleucine was used as the catalyst with 10 equivalents of water in DMSO. <sup>102,145,146</sup> They also reported that peptides and peptidic analogs with primary amino acids at their *N*-terminals can be employed as highly stereoselective catalysts. <sup>146</sup> Hayashi and coworkers reported a systematic study of the effectiveness of proteinogenic amino acids in DMSO and aqueous DMSO containing 3 equivalents of water (Table 1). <sup>103</sup> With the exceptions of phenylalanine, lysine, arginine, aspartic acid, and glutamine, in most cases the diastereoselectivity increased when the reaction was performed in aqueous DMSO. A marked increase in the diastereoselectivity was observed when proline was employed: an excellent *de* (82% *de*) was obtained in aqueous DMSO in spite of the low *de* in DMSO (20% *de*). For most of the amino acids employed, the enantioselectivity was the same for the reactions in water-free conditions and those in aqueous DMSO. Water had the positive effect of increasing the enantioselectivity only when proline, serine, and histidine were employed.

Hayashi further investigated aldol reactions of 2-butanone 298 to study the effect of water (Table 2). <sup>103</sup> Two regioisomers 299 and 300 were formed and the effect of water in these two aldol reactions was different. Water had a positive effect when the ethyl side of the ketone reacted, whereas no effect was observed when the methyl side reacted. No effect of water was observed with respect to the regioselectivity of the reaction.

## 2.07.2.3.2 Water as a solvent

In general, organocatalytic reactions are carried out in a one-pot operation by stirring a carbonyl compound, an amine, and an electrophile in conventional organic solvents, such as DMSO, DMF, or CHCl<sub>3</sub>, which are toxic, flammable, and volatile. Removal of water is not required for the formation of an enamine intermediate that proceeds to react directly with an electrophile and water tolerance is a desirable characteristic for an organocatalyst. Unlike native aldolases or aldolase antibodies, however, in the presence of bulk water aldolase-type organocatalytic reactions generally result in very poor yield and stereoselectivity. 57,115 It thus seemed

Table 1 Effectiveness of various amino acids in aldol reactions: comparison of DMSO to aqueous DMSO<sup>103</sup>

In DMSO					In DMSO-H	1 <sub>2</sub> 0			
Catalyst	Time (h)	Yield (%)	de (%)	ee (%)	Catalyst	Time (h)	Yield (%)	de (%)	ee (%)
Gly	30	73	26	0	Gly	30	75	70	0
Ala	12	74	64	90	Ala	12	72	86	90
Val	6	79	70	93	Val	6	65	88	96
lle	24	84	69	96	ile	24	84	86	97
Pro -	2	41	20	84	Pro	2	79	82	96
Ser	48	79	29	75	Ser	48	84	68	91
His	24	80	46	59	His	24	82	57	67

Table 2 Effect of Pro, Val, and Ile in aldel reactions of butanone 103

Catalyst	Solvent	299		300			
		Yield (%)	ee (%)	Yield (%)	de (%)	ee (%)	
Pro	DMSO	45	65	16	44	89	
Pro	Aqueous DMSQ	46	69	25	> 90	98	
Val	Aqueous DMSO	9	55	14	47	74	
lie	Aqueous DMSO	11	59	15	49	63	

difficult to achieve an asymmetric aldol reaction using water as a solvent, without any organic solvents or additives. It is a common misconception to consider enzymatic reactions as actually taking place 'in water.' An enzyme-catalyzed reaction might more instructively be regarded as taking place in organic solvent wherein the enzyme itself is essentially a water-soluble reaction flask that presents a stereodefined array of organic side chains that affect catalysis. As noted by the Sharpless group, the use of water as the only supporting medium for a reaction provides for ease of product isolation, high specific heat capacity, high specific inductive capacity, unique redox stability, and nonexhaustible resource, even if the rate acceleration is negligible. <sup>138</sup> Developments in aldol reactions catalyzed by enamine-based organocatalysts in aqueous media without addition of organic solvents are highlighted here. <sup>140,141,142</sup>

The pioneering work in this field has been independently reported by the Takabe and Barbas group and the Hayashi group based on two distinct strategies. Designed small diamine catalyst 301 (10 mol%) in the presence of trifluoroacetic acid in an emulsion system catalyzes the direct cross-aldol reaction of cyclohexanone (105, 2 equivalents) with 4-nitrobenzaldehyde 124 in bulk water (111 equivalents), giving the *anti*-aldol product 146 in quantitative yield with 94% *ee* (Table 3, entry 1). <sup>147</sup> A stoichiometric amount of donor 105 was enough to complete the reaction, thereby increasing the economy of the reaction (entry 2). Catalyst loading could also be decreased to 1–0.5 mol% (entries 3 and 4), <sup>140</sup> although no reaction was observed at 1 mol% catalyst loading using DMSO only as solvent. <sup>148</sup> Furthermore, crude aldol products 146 are readily isolated by removal of water using centrifugal separation; no extraction and washing are needed. The recovered catalyst 301 as well as water can be used again. The second strategy employed *trans*-1-siloxyproline 302 as the key catalyst. Using this siloxyproline catalyst 302 under a two-phase system, a marked increase in diastereoselectivity was observed when water was employed as the solvent (entries 5 and 6). <sup>39,60</sup> As these procedures use a small amount of water (3–18 equivalents), these reactions are generally called 'direct aldol reactions in the presence of water. <sup>149–151</sup> It should be noted, however, that a large excess of water does not disturb the reaction at all. The reaction proceeds smoothly even in the presence of 350 equivalents of water to provide the same excellent selectivity (entries 7 and 8). <sup>60</sup> A great number of direct aldol reactions in aqueous media with no organic solvent have now been reported. Several of these reports are summarized in Table 3. On the basis of investigations of different salting-out and salting-in conditions, brine is clearly a

unique aqueous media that accelerates the rate of reaction and affects the stereochemical outcome of the aldol reactions due to hydrophobic aggregation (entries 9 and 10). 152,153 As two electron-withdrawing ester groups strengthen the double hydrogen bonds and the siloxy group increases hydrophobicity, the catalyst 305 is highly reactive (Table 3, entry 11). 154 A combination of fluorous separations with catalysis in water is achieved by the fluorous pyrrolidine sulfonamide 306, which provides the aldol product 146 in good yield with high stereoselectivity (entry 12). 155 Fluorous extraction allows catalyst recovery and reuse for at least seven cycles. The primary-tertiary diamine 307 in the presence of triflic acid also catalyzes the aldol reaction in water (entry 13).156

Aldol reactions in aqueous media without addition of organic solvents Table 3

Catalyst 
$$H_2O$$
  $H_2O$   $H_2O$ 

Entries	Catalyst (mol%)	105 (equivalents)	H <sub>z</sub> O (equivalents)	Temp (°C)	Time (h)	Yield (%)	de (%)	ee (%)	References
1	301 (10)	2	111	25	24	99	78	94	147
2	301 (10)	ĺ	111	25	48	98	70	92	147
3	301 (1)	2	111	25	24	91	62	91	140
4	301 (0.5)	2	111	25	48	81	62	89	140
5	302 (10)	5	18	r.t.	5	86	>90	>99	59
6	302 (1)	2	3	r.t.	42	89	88	97	60
7	302 (10)	5	100	r.t.	18	84	85	>99	60
8	302 (10)	5	350	r.t.	18	77	88	>99	60
9	303 (2.5)	1.2	1114	r.t.	28	97	86	93	152
10	304 (0.5)	4	55°	<b>– 10</b>	20-48	NI <sup>b</sup>	74	91	153
11	305 (1)	2	55	25	5	99	> 98	94	154
12	306 (10)	10	111	0	9	92	66	90	155
13	307 (10)	2	111	r.t.	60	95	90	95 <sup>c</sup>	156

Brine was used as aqueous media.

Hayashi developed a direct asymmetric cross-aldol reaction of two different aldehydes in the presence of water, catalyzed by a proline-based surfactant 281 (Scheme 60). 157 Propanal 170 is water soluble, whereas 2-chlorobenzaldehyde 291 is not; thus an emulsion was formed in the reaction mixture and excellent diastereo- and enantioselectivities were attained using the surfactant catalyst 281, the length of the proline side chain dramatically affected the yield: neither very long nor very short chains were effective, whereas catalyst 281 with a decanoate moiety was found to be the most efficient. Diastereo- and enantioselectivities decreased slightly as the amount of water in the reaction was increased. The aldol reactions also proceeded efficiently under neat reaction conditions, though slight decreases in diastereo- and enantioselectivities were observed. This result provides evidence that the reaction proceeds in the organic phase, and when the reaction is performed in the presence of water, it takes place inside emulsion pockets.

Remarkable temperature-dependent properties were reported for the aqueous aldol reaction using the designed diamine catalyst 301. 140 When the direct aldol reaction is carried out in DMSO at 10 mol% catalyst loading, enantioselectivity sharply decreases as the temperature is raised (Figure 9). This observation, that ee decreases with increasing temperature, is quite general

<sup>\*</sup>Not indicated in detail.

Enantiomer of 146 was obtained as a major product.

Scheme 60 Aldehyde-aldehyde cross-aldol reaction catalyzed by a proline-surfactant organocatalyst in the presence of water.

for asymmetric syntheses in organic solvents. However, with water as solvent, the enantioselectivity is only slightly decreased at an elevated temperature and at 10 mol% catalyst loading ( $94\% \rightarrow 90\%$  ee). At 0.5 mol%, reactivity improved and enantioselectivity is maintained (yield  $52\% \rightarrow 86\%$ , ee  $89\% \rightarrow 72\%$ ). These results suggest that hydrophobic interactions play an important role in reactivity and enantioselectivity, as increased temperature leads to an increased hydrophobic effect (i.e., the entropy-driven effect).

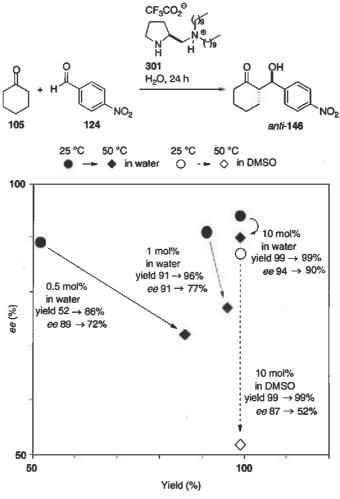


Figure 9 Temperature-dependent properties in aqueous aldol reactions.

The mechanism underlying the rate acceleration and the excellent enantioselectivity of reactions catalyzed by small amines in water is complex; a likely 'in water' mechanism is shown in Figures 10a. <sup>140,148,158</sup> A liquid organic donor assembles in water due to hydrophobic interactions, which forms a metastable micelle with the catalyst. Aggregation of the organic molecules excludes water from the organic phase and drives the equilibrium toward enamine formation. The enamine intermediate, composed of the

carbonyl donor and the catalyst, is more hydrophobic than catalyst alone; therefore, the enamine intermediate moves into the organic phase. It is believed that carbon-carbon bond formation between the enamine intermediate and the aldehyde acceptor occurs quickly in the highly concentrated organic micellar phase through a transition state similar to that observed in organic solvents, and then hydrolysis of the enamine intermediate proceeds. An 'on water' mechanism has also been proposed. <sup>159,160</sup> In this mechanism, a free hydroxy group at the oil-water phase boundary protrudes into the organic phase to catalyze reactions through the formation of hydrogen bonds to a donor, an acceptor, and/or the catalyst (Figure 10b).

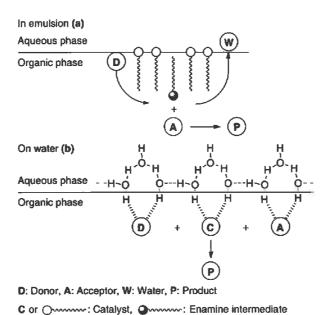


Figure 10 Cartoons of (a) in emulsion and (b) on water catalysis.

As both lipophilic and surfactant organocatalysts can promote asymmetric direct aldol reactions with excellent enantioselectivity in two-phase or emulsion systems, it was previously thought that they proceed in the organic phase at least at its surface. However, aldol reactions in which both the substrate and the catalyst dissolve homogeneously in water have been reported. The Hayashi group reported that some amino acids, dipeptides, and amides derived from amino acids proceed the self-aldol reaction of propanal 170 in water (Table 4). 161 Notable result was obtained when Pro-NH<sub>2</sub> 129 was utilized. A moderate yield was

Table 4 Effect of amino acid, amino amide, or dipeptide as catalysts for the self-aldol reaction of propanal in water<sup>161</sup>

Entries	Catalyst	Time (h)	Yield (%)	dr (anti/syn)	ee (%)	
					anti	syn
1	Ala	24	<3	ND	ND	ND
2	Pro	24	<3	ND	ND	ND
3	Arg	24	15	64:36	-18	-14
4	Ala-Ala	24	15	50:50	18	3
5	Ala-NH <sub>2</sub>	24	18	45:55	28	11
6	Tyr-NH <sub>2</sub>	24	41	48:52	-53	-13
7	Thr-NH <sub>2</sub>	24	32	40:60	13	9
8	Pro-NH <sub>2</sub>	3	41	48:52	78	74

ND, not determined.

obtained in short reaction time with good enantioselectivities (anti 78% ee, syn 74% ee). As both propanal and Pro-NH<sub>2</sub> 129 dissolve completely in water, this aldol reaction proceeds 'in water' and not 'in the organic phase,' at least for the first 2.5 h.

In these reactions catalysed by organocatalysts, there are several discussions and discrepancies about the role of water and the appropriate terminology to use. 149-151 Hayashi proposed to use the term 'in water' when the reactants participating in the reaction are homogeneously dissolved whereas the term 'in the presence of water' should be used for a reaction that proceeds in a concentrated organic phase with water being present as a second phase and influencing the reaction in the organic phase. Although the observed effect for 'on water' reactions is rate acceleration, the observed effect for 'in the presence of water' reaction is an increased enantioselectivity. The reasons behind yield improvement are different in each reaction system. Some case would be effected by 'on water,' or accelerate phase-transfer catalyst. However, it should be noted that there is known organocatalyzed aldol reaction in homogeneous aqueous solution in the case of prolinamide 129-catalyzed self-aldol reaction of propanal 170 (Table 4). Meanwhile, water improves diastereoselectivities and enantioselectivities by some kind of interaction, the reason behind this influence is still unclear. These reactions may be predisposed to favor transition states that optimize hydrophobic interactions, when highly hydrophobic catalysts are employed in the presence of water. 162,163

## 2.07.2.4 Multicatalytic Reaction Sequences

## 2.07.2.4.1 Metal-catalyzed and organocatalytic reaction sequences

In recent years, combinations of transition metal catalysis and organocatalysis have attracted increasing attention in synthetic organic chemistry. These combinations allow for the regio- and stereoselective synthesis of complex molecule from readily available, simple starting materials. <sup>164–167</sup> A one-pot Rh complex-catalyzed hydroformylation, following (S)-proline-catalyzed enantioselective cross-aldol reaction sequence, is one example (Table 5). <sup>168</sup> Simple alkenes 310 are transformed *in situ* to aliphatic aldehydes 311, giving aldol products 312 in good yields with excellent stereoselectivities in a one-pot operation.

Table 5 Metal-catalyzed and organocatalytic reaction sequence 68

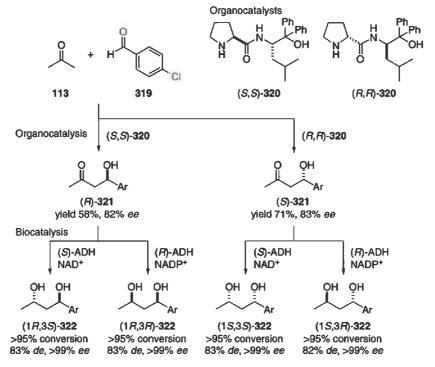
Entries	R1	R <sup>2</sup>	6 (mol%)	[Rh] (mot%)	Ligand (mol%)	Conditions	Yield (%)	de (%)	ee (%)
1	Н	<i>i</i> -Pr	6	0.2	314 (4.4)	30 bar, 5 °C	76	90	98
2	Н	<i>c</i> Hex	5	0.25	314 (5.0)	30 bar, 5 °C	81	86	99
3	н	Ph	5	0.25	315 (1.0)	30 bar, 5 °C	91	50	94
4	Hex	<i>i</i> -Pr	0.15	0.01	<b>315</b> (0.1)	20 bar, 15→5 °C	86	90	97

# 2.07.2.4.2 Organocatalytic and biocatalytic reaction sequences

Like combinations of metalcatalysts and organocatalysts in Table 5, combining biocatalysis and organocatalysis is a powerful strategy to improve the synthetic efficiency. As (S)-proline has an insignificant effect on the lipase-catalyzed reaction, the one-pot sequential and/or tandem reactions are investigated by the Córdova group (Scheme 61). Although the first (S)-proline-catalyzed aldol reaction provides the desired aldol 317 with less than 80% ee, excellent enantioselectivities are achieved after the second lipase-catalyzed kinetic resolution in reasonable chemical yields.

Scheme 61 Sequential direct aldol reactions and lipase-catalyzed kinetic resolutions.

Sequential two-step synthesis based on the organocatalytic aldol reaction and biocatalytic reduction leads to all four possible stereoisomers of 1,3-diols 322 in enantiomerically pure form (Scheme 62). The stereochemistry depends on the combination of the organocatalyst 320 and alcohol dehydrogenase. Furthermore, conversion is improved up to 80% by sequential one-pot synthesis without the workup step after the organocatalytic aldol reaction.



Conditions: organocatalyst 320 (5 mol%), r.t., 18 h biocatalyst, 2-PrOH, Buffer, r.t., 18-67 h

Scheme 62 Organocatalytic and biocatalytic reaction sequence.

# 2.07.2.5 Mechanistic Studies

A number of experimental and theoretical studies have been carried out in order to elucidate the mechanism of the (S)-proline-catalyzed aldol reaction. The catalytic cycle of (S)-proline-catalyzed aldol reactions of nucleophilic aldehydes and ketones with carbonyl electrophiles as shown in Scheme 63 is widely accepted by the organic chemists, in spite of being still under debate.

Nucleophilic attack of the nitrogen atom of (S)-proline 6 to the starting carbonyl compound 1 affords an iminium intermediate 324 through dehydration of an aminal intermediate 323. The iminium 324 and oxazolidinone 325 are the substances at equilibrium. Nucleophilic enamine 326 is formed through deprotonation, making carbon-carbon bond together with the carbonyl compound 228 as an electrophile. This step is the rate-determining step, and possible transition states are given later. The alkylated iminium 327 and/or oxazolidinone 328 are hydrolyzed to give an aminal intermediate 329. Finally, the desired aldol 330 is released from 329 and (S)-proline 6 as a catalyst is regenerated. Overall, this mechanism is similar to the known type I aldolase mechanism as noted in Figure 3.

Scheme 63 Catalytic cycle of aldol reactions with (S)-proline.

Seebach and Eschenmoser proposed another mechanism (Scheme 64). <sup>171</sup> Carbonyl compound 1 reacts with (S)-proline to afford oxazolidinone 325, from which enaminocarboxylic acid is generated. From this enamine, with the C=C bond of (E)-configuration and the C-atom bearing the COOH group in an s-cis-arrangement with the enamino C=C bond, carboxylate anion attacks the double bond of enamine 332. Next, the generated anion further attacks the electrophile from the opposite side of the carboxyl moiety to afford the oxazolidinone derivative 333, which is hydrolyzed to provide the desired product 330.

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

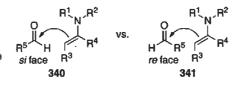
$$\begin{bmatrix}
R_1 & CO_2H \\
R^2 & H & H
\end{bmatrix}$$

Scheme 64 Another mechanism proposed by Seebach and Eschenmoser.

The efficiency of aldol reactions is expected to be influenced by the rate of the formation of enamine, the addition step, and stability of catalyst, among other factors. The carbon-carbon bond-forming step has a similar energy barrier as the enamine formation, indicating that under different conditions or with different substrates, the rate-determining step may be this step. In fact, the most recent kinetic evidence obtained by Armstrong and Blackmond indicates that under the reaction conditions studied, the addition step is rate determining. <sup>172</sup> It is believed that four factors control the stereoselectivity of the product (Figure 11): (1) the geometry about the C-N bond of the enamine (334 vs. 335); (2) the geometric isomer of the formed enamine (336 vs. 337); (3) the enantiotopic face of the enamine for C-C bond formation (338 vs. 339); and (4) the enantiotopic face of the electrophile for C-C bond formation (340 vs. 341). Several catalysts have been developed to address and to control these issues.

Understanding a reaction mechanism and transition state is very important to realize the whole reaction and stereochemistry. Several reaction intermediates and transition state models are previously accepted in intramolecular aldol reaction (Figure 12). Achiral secondary amines such as pyrrolidine and piperidine have been used as a catalyst for intramolecular aldol reaction since its discovery in 1950 reported by Wieland and Miescher 173; after a decade and a half, the Spencer group confirmed enamine

 The geometry about the C-N bond of the enamine



- The enantiotopic face of the enamine for C-C bond formation
- The enantiotopic face of the electrophile for C–C bond formation

Figure 11 Four factors that affect stereoselectivity in aidol additions.

intermediates 342 through mechanistic studies.<sup>174</sup> The Hajos group showed two possible mechanisms for (S)-proline-catalyzed intramolecular aldol reaction in 1974.<sup>5</sup> The first is enamine mechanism 343a including oxazolidinone mechanism 343b, which was recently discussed again by the Seebach and Eschenmoser group.<sup>171</sup> However, enamine mechanism 343a is controverted, because no incorporation of <sup>18</sup>O into the aldol product 37 is observed in (S)-proline-catalyzed intramolecular aldol reaction of triketone 35 in the presence of <sup>18</sup>O-labeled water. Thus, they propose enol mechanism 343c, which involves the addition of (S)-proline in its zwitterionic form to one of the carbonyl groups of the cyclopentanedione ring.

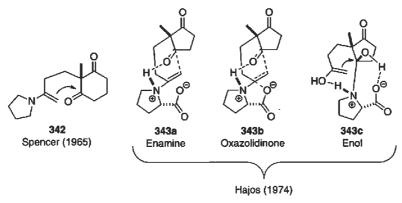


Figure 12 Proposed reaction intermediates and transition state models.

The Hajos group has proposed the enol mechanism according to their <sup>18</sup>O-labeled experiments; however, the List group recently reexamined it to prove the enamine mechanism (Scheme 65), <sup>175</sup> though detection of enamines derived from aldehydes or ketones with (S)-proline is still difficult to achieve. A high <sup>18</sup>O incorporation (>90%) is observed: (1) when the reactions are performed under completely air- and moisture-free conditions; (2) when both the substrate and proline catalyst are carefully dried azeotropically; and (3) when dried solvent (DMSO) is used. These results support that the final hydrolysis step from the iminium 346 to the ketone 347 requires <sup>18</sup>O-enriched water, namely, the mechanism of the Hajos–Parrish–Eder–Sauer–Wiechert reaction is confirmed as the enamine mechanism not enol mechanism.

Dilution effect and nonlinear effect indicate that the catalysis for intramolecular aldol reaction needs more than one proline molecule per triketone molecule substrate reported by the Kagan and Agami group (Figure 13, 349).<sup>176,177</sup> As the computational chemistry is developed, the mechanism for (S)-proline-catalyzed aldol reaction is investigated. The Houk group shows evidence for the involvement of only one proline molecule in the transition states of intramolecular aldol reactions, which are based on kinetic measurements and the absence of nonlinear and dilution effects. In addition, these are supported by B3LYP/6-31 G<sup>+</sup> calculations. T8-182 In the chair transition state model 350a leading to the corresponding aldol (S)-37, hydrogen bonding between the carbonyl group of electrophile and the carboxylic acid of (S)-proline is formed. The favorable electrostatic interaction of T8-NCH- O<sup>6-</sup> also contributes to the lower energy of transition state 350a. Similarly, a Zimmerman-Traxler chair-like model 350b is also proposed for (S)-proline-catalyzed intermolecular aldol reaction. Double activation by (S)-proline as a bifunctional

Scheme 65 Proposed enamine catalysis cycle of the Hajos-Parrish-Eder-Sauer-Wiechert reaction.

catalyst is found again, that is, (1) activation of nucleophile through enamine formation with pyrrolidine moiety and (2) activation of electrophile through hydrogen bonding with Brønsted acid moiety.

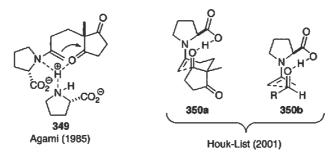


Figure 13 Proposed transition state models.

#### 2.07.3 Brønsted Acid and Hydrogen-Bond Catalysis

Asymmetric reactions catalyzed by chiral Brønsted acids have become the subject of cutting-edge research in synthetic organic chemistry in recent years. [183+187] There are three modes of activation of carbonyl compounds (Figure 14): (1) Brønsted acid catalysis 351, (2) double hydrogen bonding 352, and (3) single hydrogen bonding 353. It is of note, however, that there is no clear distinction between Brønsted acid catalysis and hydrogen-bond catalysis.

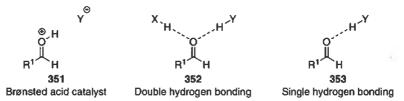


Figure 14 Modes of activation for Brønsted acid and hydrogen-bond catalysis.

In general, the activation of the substrate is enabled through hydrogen bonding or formation of ion pairs, depending on the acidic strength of the catalyst such as phosphoric acids, <sup>188–195</sup> ammonium salts, thioureas, <sup>196</sup> diols, <sup>197</sup> and squaramides. <sup>198</sup> Among the different chiral Brønsted acids (Figure 15), axial chiral phosphoric acid derivatives have shown astonishing versatility.

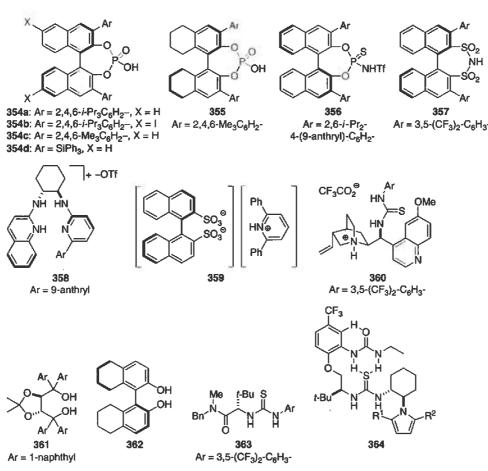


Figure 15 Representative Bransted acid and hydrogen-bond catalysts.

## 2.07.3.1 Intermolecular Aldol Reactions in Brønsted Acid and Hydrogen-Bond Catalysis

#### 2.07.3.1.1 Aldoi-type reactions of aziactone

The direct aldol-type reaction of azlactone 365 with an oxocarbenium ion through a protonation of vinyl ethers 366 by the chiral phosphoric acid 354a as a catalyst provides  $\beta$ -alkoxy- $\alpha$ -amino acid derivatives 367 bearing a quaternary stereogenic center with high diastereo- and enantioselectivity (Scheme 66). 199

354a (5 mol%) 
$$CH_2Cl_2$$
, 0 °C or r.t. MeONa  $MeOH$   $Ar^2$   $Ar^1$  365 366  $Ar^1 = 3.5$ -(MeO)<sub>2</sub>-C<sub>6</sub>H<sub>3</sub>  $Ar^2$   $Ar^3$   $Ar^4$   $Ar^5$   $Ar^5$   $Ar^5$   $Ar^6$   $Ar^6$ 

Scheme 66 Direct aldol-type reactions of azlactone.

The ion pairs 368 or 369 likely have a three-dimensionally oriented specific interaction between a chiral conjugate base and an oxocarbenium ion through a C-H···O hydrogen bonding model, controlling enantioselectivity of the transformations (Figure 16).

# 2.07.3.1.2 Mukaiyama aldol reactions in Brønsted acid catalysis

The Mukaiyama aldol reaction is one of the most synthetically reliable carbon-carbon bond-forming reactions.<sup>3</sup> Although a wide variety of Lewis acid and Lewis base catalysts have been developed for enantioselective Mukaiyama aldol reactions involving silyl

C-H---O hydrogen bonding model

Figure 16 Reactive intermediate in direct aldol-type reactions of azlactone.

enol ether as a stable enolate component, very few examples of chiral Brønsted acid-catalyzed Mukaiyama aldol reactions were reported before 2006. Catalyst loading (10 mol%) may not be sufficient due to their lower acidities.  $^{200,201}$  To increase the reactivities of chiral phosphoric acid catalysts, chiral *N*-triflyl thiophosphoramide 356 was developed to catalyze the Mukaiyama aldol reactions of aldehydes 24 using silyl enol ethers of ketones 370 as nucleophiles (Scheme 67–1). From mechanistic studies, the type of catalysis depends on reaction temperature: (1) a Lewis acid pathway associated with the silylated Brønsted acid occurs at room temperature and (2) a Brønsted acid pathway associated with the Brønsted acid itself takes place at a low temperature.  $^{202}$  A designed chiral disulfonimide 357 was developed as a powerful Brønsted acid for the Mukaiyama aldol reaction of aldehydes 24 using silyl enol ethers of esters 372 as nucleophiles; these reactions provide the desired aldol products 373 with more than 90% *ee* in most cases (Scheme 67–2). The actual catalyst is proposed to be an *in situ* generated *N*-silyl imide.  $^{203}$  An excellent contribution was made by Rawal and coworkers in 2003 who developed a chiral Brønsted acid catalyst, TADDOL ( $\alpha,\alpha,\alpha',\alpha'$ -tetraaryl-2,2-dimethyl-1,3-dioxolane-4,5-dimethanol) in hetero-Diels-Alder reactions.  $^{204}$  They also developed that a simple chiral diol of the TADDOL 361 catalyzes Mukaiyama aldol reactions between aldehydes and silyl enol ethers of amides 374.  $^{200,201}$  In addition, hydrogen-bond activation by the diol catalyst 361 is applicable for the Mukaiyama aldol reactions of acyl phosphonates 375 with silyl enol ethers of amides 374, giving the aldol product 376 with one tertiary and one quaternary stereocenter with excellent diastereo- and enantioselectivities (Scheme 67–3).

Scheme 67 Mukaiyama aldol reactions.

The applications of aldol reactions with \(\alpha\_i\)-unsaturated carbonyl compounds as nucleophiles, in general, called vinylogous aldol reactions, have attracted much attention. 200 Rawal developed enantioselective vinylogous Mukaiyama aldol reactions of 377 and 378 using 361 in 2005 (Scheme 68). 207 TADDOL catalysts are reliable because they generally exist in a well-defined internally hydrogen-bonded arrangement; furthermore, these catalysts are most effective with reactive aldehydes. In such reactions, the proposed mechanism proceeds through activation of the aldol acceptor 378 by hydrogen bonding.

Organocatalytic approaches using a wide variety of Brønsted acids and hydrogen-bond catalysts have been intensively investigated after 2005. The silyloxy furans 380 are good substrates for the vinylogous Mukaiyama aldol reaction; however, their applications have been limited until recently. Carboxylate-ammonium salt 360 prepared from a cinchona-thiourea and trifluoroacetic acid is an effective catalyst for the vinylogous aldol reaction of the silyloxy furans 380 with aldehydes 228, producing anti-aldol products 381 with high enantiomeric excess (Scheme 69-1). 209 Like the Mukaiyama aldol reaction (Scheme 67-2), the

Scheme 68 TADOOL-catalyzed vinylogous Mukaiyama aldol reaction.

TMSO 
$$R^2$$
  $R^3$   $R^3$ 

Scheme 69 Vinylogous Mukaiyama aldol reactions.

vinylogous Mukaiyama aldol reactions of crotonic acid-derived nucleophiles 382 with aldehydes 24 are catalyzed by the disulfonimide 357. Extremely high regioselectivities in  $\gamma/\alpha$  ratios up to > 40:1 and more than 90% enantiomeric excesses are observed in most cases (Scheme 69-2).<sup>210</sup> This methodology was further expanded to  $\varepsilon$ -selective double vinylogous Mukaiyama aldol reactions (Scheme 69-3).<sup>210</sup> The single-step syntheses of chiral  $\alpha,\beta$ - and  $\alpha,\beta,\gamma,\delta$ -unsaturated esters 383 and 385 through vinylogous and double vinylogous Mukaiyama aldol reactions would be potentially useful for natural product synthesis.

#### 2.07.3.1.3 Direct aldol reactions in Brønsted acid catalysis

Blanchet reported phosphoric acid-catalyzed direct aldol reactions between ketones 386 and ethyl glyoxylate 387, which is a reactive electrophile (Scheme 70).<sup>211</sup> Moderate to excellent diastereo- and enantioselectivities have been achieved using H<sub>8</sub>-BINOL-derived phosphoric acid 355. The aldol products 388 have *syn*-configurations; thus, this reaction is complementary to

Scheme 70 Phosphoric acid-catalyzed direct aldol reactions

#### 320 The Aldol Reaction: Organocatalysis Approach

(S)-proline catalysis in Brønsted acids, which in general yields the *anti*-configuration. Furthermore, unreactive substrates in enamine organocatalysis were able to afford the aldol products under these conditions.

#### 2.07.3.2 Intramolecular Aldol Reactions in Brønsted Acid Catalysis

Significant desymmetrizations of meso-1,3-diones 389 result from intramolecular aldol reactions catalyzed by chiral phosphoric acid 354a. This method affords a wide variety of chiral cyclohexenones 390 in high yields and with excellent enantioselectivities carried out by the Akiyama group (equation 16).<sup>212</sup>

ONIOM (B3LYP/6-31 G': HF/3-21 G) calculations suggest the identity of the reaction intermediate and the origin of the enantioselectivity (Figure 17). <sup>212</sup> In the transition state model 391, the carbonyl and enol groups are activated by chiral phosphoric acid 354a with Brønsted acidic and Lewis basic sites, respectively. The attack from the *si* face is more favorable than attack from the *re* face and provides the (*R*)-isomer 390 (R<sup>1</sup>=Me). As the (*S*)-isomer is formed in the (*S*)-proline-catalyzed Hajos-Parrish-Eder-Sauer-Wiechert cyclization through transition state 392, <sup>4-7</sup> Brønsted acid catalysis and enamine catalysis are complementary methodologies.

Figure 17 Proposed intermediates in aidol reactions comparing phosphoric acid catalyst to (S)-proline.

#### 2.07.4 Brønsted Base Catalysis Including Bifunctional Catalysis

# 2.07.4.1 Aldol Reactions in Brønsted Base Catalysis Including Bifunctional Catalysis

Organic Brønsted bases have been used to catalyze synthetically valuable carbon-carbon bond-forming reactions of carbonyl and related compounds for decades. With the development of organocatalysis, chiral Brønsted base catalysis has generated significant interest. <sup>213</sup> Nitrogen-containing compounds such as tertiary amines, guanidines, <sup>214,215</sup> amidines, and imidazoles are predominantly used as a chiral Brønsted base catalyst; in particular, the cinchona family is effective due to their extensive versatility, stereochemical diversity, and commercial availability. Bifunctional catalysts such as tertiary amine-thiourea, cinchona-thiourea, and guanidine-thiourea are important in organocatalysis<sup>216,217</sup>; they are included in this section because reactive intermediates in bifunctional catalysis are often onium enolates or enolate-like species, similar to Brønsted base catalysts.

Recently, direct vinylogous aldol reactions of furanones 393 with aldehydes or ketones 3a have been intensively investigated. These reactions are simple alternatives to the vinylogous Mukaiyama aldol reaction of silyloxy furans discussed in Scheme 69. Highly enantioselective direct vinylogous aldol reactions of dihalogenated furanones 393 ( $R^1=R^2=Cl$ ) with aldehydes 3a ( $R^3=H$ ) are catalyzed by the axially chiral guanidine 395. The substituent attached to the guanidine moiety significantly influences the diastereo- and enantioselectivity as well as the catalytic activity. A bis(3,4,5-trimethoxyphenyl)methyl group shows the best results (Table 6, entries 1 and 2). <sup>218</sup> *y*-Substituted butenolides 394 containing a tertiary alcohol moiety are provided by the highly *syn*-selective vinylogous aldol reactions between furanones 393 and ketoesters 3a ( $R^3=Ph$ ,  $R^4=CO_2tBu$ ) catalyzed by the bifunctional catalyst 396 containing tertiary amine, thiourea, and tryptophan moieties (entry 3). <sup>219</sup> The *anti*-selective vinylogous aldol reactions of the unactivated furanones 393 ( $R^1=R^2=H$ ) with aldehydes 3a are achieved by the use of cinchona-thiourea bifunctional catalyst 397 under mild conditions (entry 4). <sup>220</sup> Similarly, bifunctional tertiary amine-squaramide 398 enantio-selectively catalyzes the vinylogous aldol reaction, but prolonged reaction times are necessary (entry 5). <sup>221</sup>

The proposed transition state 399 in this syn-selective vinylogous aldol reaction is shown in Figure 18. A guanidium ion, generated from the deprotonation of the furanone derivative 393 by the guanidine catalyst 395, would interact not only with the anion but also with aldehyde 3a through hydrogen-bonding interactions.<sup>218</sup>

#### Table 6 Direct vinylogous aidol reactions in Brønsted base catalysis

Entries	R1	R <sup>2</sup>	R³	R <sup>4</sup>	Catalyst (mol%)	Conditions	Yield (%)	dr (anti/syn)	ee (%)³	References
1	CI	ÇI	Н	Ph	395 (5)	THF, -40 °C, 5 h	90	23:77	99	218
2	Br	Br	Н	4-MeC <sub>6</sub> H₄	395 (10)	THF, -40 °C, 12 h	95	14:86	99	218
3	CI	CI	Ph	CO2t-Bu	396 (10)	Acetone/THF (1:1), r.t., 24 h	88	3:97	93	219
4	Н	н	Н	Ph T	397 (10)	Et <sub>2</sub> O, 30 °C, 50 h	87	85:15	82 <sup>b</sup>	220
5	Н	Н	Н	2-Naphthyl	398 (20)	CH <sub>2</sub> Cl <sub>2,</sub> r.t., 10 days	73	86:14	95 <sup>b</sup>	221

<sup>&</sup>lt;sup>a</sup>The *ee* is indicated for the major product.

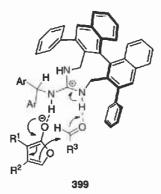


Figure 18 Proposed transition state model in the syn-selective vinylogous aldol reaction.

The chiral bicyclic guanidine 401 acts as a Brønsted base catalyst. The hydroxy group controls diastereo- and enantioselectivities in the direct aldol reaction of 5*H*-oxazol-4-ones 400 with aldehydes 224. The aldol products 402 are easily converted to amides or esters without loss of enantiopurity; thus, this method provides synthetically useful  $\alpha,\beta$ -dihydroxycarboxylates bearing a chiral quaternary stereogenic center at the  $\alpha$ -carbon atom (Scheme 71).

Several groups have suggested that protonated cinchona alkaloids activate electrophiles by hydrogen-bond donation.<sup>223</sup> Among the different cinchona alkaloid derivatives tested, the enantioselective aldol reaction between 1,3-cyclohexanedione 403 and various  $\alpha$ -bromoketoesters 404 followed by cyclization gave the bicyclic product 408 (Scheme 72).<sup>224</sup> The dimeric catalyst 405 showed the best results when a proton sponge 406 and an ammonium salt were used in the reaction mixture.

The alternative rotamer of the hydrogen-bonded intermediate 410, which would favor si face attack and formation of the opposite enantiomer, is likely to be disfavored by interactions between the bromine of the substrate and the pyrimidine ring of the catalyst (Scheme 73).

Shibata, Toru, and coworkers reported a cinchona alkaloid-catalyzed aldol reaction of oxindoles 411 with trifluoropyruvate 412 (Scheme 74).<sup>225</sup> (DHQD)<sub>2</sub>PHAL 413 and (DHQ)<sub>2</sub>PHAL afforded the best results. By employing suitable cinchona alkaloids as

Enantiomer ((5R,1'S)-ent-anti-394) was obtained.

# 322 The Aldol Reaction: Organocatalysis Approach

Scheme 71 Guanidine base-catalyzed aldol reaction of 5H-oxazol-4-ones.

Scheme 72 Cinchona alkaloid-catalyzed aldol/cyclization.

Scheme 73 Mechanism of a cinchona alkaloid-catalyzed aldol reaction process.

catalysts, both enantiomers 414 with two contiguous asymmetric quaternary carbon centers can be obtained. The CF<sub>3</sub> group of the pyruvate is essential for success of this type of aldol reaction.

Aldol reactions of unactivated ketones 415 with isatins 416 are catalyzed by the quinidine-thiourea bifunctional catalyst 417 through an ammonium enolate mechanism. The enolate of ketone 415 closely associates with the catalyst 417 through ionic interactions; in addition, two carbonyl groups of isatin 416 are activated by two hydrogen bonds with the thiourea moiety of catalyst 417 as shown in the transition state model 419 (Scheme 75).<sup>226</sup> The reaction results in high yields and enantioselectivities.

414d (yield 97%)

78% de, 96% ee

$$\begin{array}{c} & \begin{array}{c} & \begin{array}{c} 413 \text{ (10 mol\%)} \\ & \begin{array}{c} F_3C \\ \end{array} \\ \end{array} \\ \begin{array}{c} OH \\ CO_2Et \\ \end{array} \\ \end{array} \\ \begin{array}{c} A11 \\ \end{array} \\ \begin{array}{c} A13 \\ \end{array} \\ \begin{array}{c} CO_2Et \\ \end{array} \\ \begin{array}{c} A13 \\ \end{array} \\ \begin{array}{c} A13 \\ \end{array} \\ \begin{array}{c} A13 \\ \end{array} \\ \begin{array}{c} A14 \\ \end{array} \\ \begin{array}{c$$

414b (yield 99%)

80% de, 95% ee

414c (yield 90%)

88% de, 95% ee

Scheme 74 Cinchona alkaloid-catalyzed aldol reactions of exindole with ethyl trifluoropyruvate.

414a (yield 75%)

70% de, 95% ee

Scheme 75 Cinchona-thiourea-catalyzed aldol reactions of isatins.

# 2.07.4.2 Aldol-Lactonization Reactions in Nucleophilic Base Catalysis

 $\beta$ -Lactones are important molecules that are integral structural features of many pharmacologically active compounds, and these are very useful for synthetic intermediates. <sup>227</sup> In 1966, Borrmann and Wegler first synthesized optically active lactones 422 through formal [2+2] cycloaddition processes, consisting of  $\alpha$ -chlorinated aldehydes 421 such as chloral, (–)-brucine, and acyl halides 420, through the intermediary of an *in situ* generated ketene (Scheme 76). <sup>228–230</sup> These lactones 422 were isolated in good yields with up to 72% *ee*.

The proposed mechanism for the reaction above involves an aldol-lactonization process (Scheme 77). The chiral tertiary amine attacks ketene 424, the resulting zwitterionic ammonium enolate 426 attacks aldehyde 228, and then cyclization of the newly formed alkoxide onto the acyl ammonium species 427 forms the  $\beta$ -lactone 428. In Section 2.07.4.2, formal [2+2] sequences that involve ammonium enolates in intramolecular aldol-lactonization reactions will be discussed. 227

The Wynberg \(\beta\)-lactone synthesis was one of the first practical, catalytic, asymmetric reactions, \(^{231,232}\) in which quinidine and quinine were found to be good catalysts for the formal [2+2] cycloaddition of ketene 429 and chloral 430 (Scheme 78).

#### 324 The Aldol Reaction: Organocatalysis Approach

Scheme 76 The first example of an organocatalytic aldol-factonization reaction.

Scheme 77 Proposed mechanism in the Borrmann and Wegler example involves an aldol-lactonization process.

The resulting  $\beta$ -(trichloromethyl)- $\beta$ -propiolactone 432 was obtained in good yield and excellent enantioselectivity. Wynberg's procedure for the asymmetric synthesis of  $\beta$ -lactones was then extended by the Romo group to involve an *in situ* generated ketene with dichlorinated aldehydes. <sup>233</sup> The use of toluene as the solvent to precipitate the hydrochloride salt of Hunig's base, generating the nucleophilic free base catalyst, was crucial for the success of this reaction.

Scheme 78 Quinidine-catalyzed aldol-lactonization reactions of ketenes.

Intramolecular aldol-lactonization reactions of the aldehyde-carboxylic acid 433 catalyzed by O-acetylquinidine 435 have been developed (Scheme 79). 234 Aldehyde-acid substrates 433 in conjunction with Mukaiyama's reagent 434 as carboxylate activators, as well as catalytic amounts of the cinchona alkaloid derivative 435, delivered bicyclic \(\beta\)-lactones 436 with high enantiomeric excess and in good yield.

Scheme 79 Q-acetylquinidine-catalyzed aldol-lactonization reactions.

The Calter group reported that trimethylsilylquinine 438 catalyzed the dimerization of monosubstituted ketenes generated in situ from the reaction of acid chlorides 437 and diisopropylethylamine yields ketene dimers 439 in high yields and enantioselectivities (Scheme 80). 215 For determining yield and enantiomeric purity, the researchers immediately converted the volatile and unstable methylketene dimers into  $\beta$ -ketoamides 440. Kinetic studies suggested that the rate-determining step for the reaction is the deprotonation of the acid chloride by the tentiary amine to form the ketene.

Scheme 80 Trimethylsilylquinine-catalyzed dimerization of monosubstituted ketenes.

Deviating slightly from tertiary amine systems, chiral phosphine-catalyzed formal [2+2] cycloadditions of ketenes 441 and aldehyde 228 have also been reported (Scheme 81). 236 The BINAPHANE 442 catalytic system displayed excellent enantioselectivity and high diastereoselectivity in generating trans-isomer 443.

326

Scheme 81 Phosphine-catalyzed formal [2+2] cycloadditions.

Two mechanisms have been suggested to rationalize the observed stereoselectivity (Scheme 82).<sup>236</sup> The first mechanism A involves an attack of BINAPHANE 442 to aldehyde 228 to give phosphonium alkoxide 444, which would then add to ketene 441 to generate enolate 445. Intramolecular S<sub>N</sub>2 displacement would provide *trans* β-lactone 443 as the major product. The other mechanism B involves a BINAPHANE 442 attack on ketene 441 first, to generate enolate 446, which would then add to aldehyde 228 and go on to product 443. Although the more commonly encountered mechanism B cannot be ruled out, Kerrigan and coworkers speculate that the mechanism for the formation of *trans*-443 involves initial attack of BINAPHANE 442 on aldehyde 228 to give phosphonium alkoxide 444 through mechanism A. There is precedence for such a mode of addition in the work of Fu on the synthesis of *trans*-β-lactams from ketoketenes and N-triflyl imines.<sup>237</sup> Fu group provided both <sup>1</sup>H NMR and X-ray crystallographic evidence that the nucleophilic catalyst, a chiral 4-(pyπolidino)pyridine derivative, attacks first the N-triflylimine rather than the ketene.

**Scheme 82** Proposed mechanisms for phosphine-catalyzed formal [2+2] cycloaddition.

Curran and coworkers used urea derivatives as aldol catalysts,  $^{239}$  whereas Jacobsen and coworkers described a parallel library approach to the discovery of urea catalyst derivatives.  $^{239}$  The Takemoto group developed a novel bifunctional thiourea catalyst bearing a tertiary amine moiety.  $^{240}$  Ever since these seminal reports, chiral thiourea catalysis has been one of the growing fields in hydrogen bond catalysis. Seidel and coworkers reported aldol reactions of  $\alpha$ -isothiocyanatoimides 448 to aldehydes 122 using bifunctional thiourea catalyst 449.  $^{241}$  The catalyst loading can be even reduced to 5 mol%. Several electron-rich and poor aromatic aldehydes 122 with different substitution patterns gave rise to products 450 that were generally obtained in good yields, and with high diastereo- and enantioselectivities. The obtained products 450 are synthetic equivalents of  $syn-\beta$ -hydroxy- $\alpha$ -amino acids, which are useful intermediates for the synthesis of various natural products (Scheme 83).

Scheme 83 Bifunctional amine-thiourea-catalyzed aldol-cyclization reactions.

#### 2.07.5 Phase-Transfer Catalysis

Phase-transfer catalysis is critical not only to industrial manufacturing of high-performance chemical products in low-cost processes but also to academic research on various asymmetric bond formation reactions under mild conditions. For example, phasetransfer catalysis has been used to catalyze epoxidations, Darzens condensations, Michael reactions, Robinson annulations, and alkylations. Beneficial properties of phase-transfer catalysis such as simple operation, mild conditions, inexpensive reagents and solvents, and large-quantity preparations could open the way to a seamless development of synthetic methods from academia to industry. 242-248

#### 2.07.5.1 Aldol Reactions in Phase-Transfer Catalysis

In 1984, the first efficient chiral phase-transfer catalyst, N-(4-(trifluoromethyl)benzyl)cinchonidium bromide, was developed by the Merck group for asymmetric methylation of 6,7-dichloro-5-methoxy-2-phenyl-1-indanone, providing the desired product in 92% ee. 249 The reaction of glycine derivatives with various aldehydes using cinchonidium salt as a catalyst was the first example of aldol reaction in phase-transfer catalysis. The generated products,  $\beta$ -hydroxy  $\alpha$ -amino acid derivatives, are constituents of many bioactive peptide natural products. In 1991, Miller reported low diastereoselectivities and very low enantioselectivities in aldol reactions using cinchonidium salt 451 as a catalyst (46-92% yield, 0-56% de, 3-12% ee, Figure 19). 250 The reaction mechanism of this quaternary ammonium salt-catalyzed process is thought to proceed through an ion-pair mechanism (Figures 1, 10b). Since then, several catalysts have been reported for this reaction, but the enantioselectivities of these processes remain low. For example, cinchonidium salts 452 gave moderate results (34-78% yield, 0-14% de, 52-83% ee, Figure 19).251

Figure 19 Types of cinchonidium salt catalysts.

A significant improvement in quaternary ammonium salt-catalyzed aldol reactions was reported by Maruoka and coworkers. Highly enantioselective and anti-selective aldol reactions could be performed in the presence of 2 mol% of chiral C2-symmetric quaternary salt catalyst 455 along with NaOH and toluene (Scheme 84). An echanistic investigations revealed the intervention of an unfavorable yet inevitable retro-aldol process involving the chiral catalyst. On the basis of this information, a reliable procedure has been established; the use of 1% NaOH (aqueous) and ammonium chloride 455. Furthermore, the spiro-type phase-transfer catalyst (456, Ar=H) possessing a  $C_2$ -symmetry axis provides a single type of asymmetric environment; in contrast, a newly designed spiro-type phase-transfer catalyst (456, Ar $\neq$ H) has two different asymmetric environments. The substituents of the binaphthyl subunits affect enantioselectivity, and the 3,5-bis[3,5-bis(trifluoromethyl)phenyl]phenyl group is the best substituent of those evaluated in the anti-selective aldol reactions of glycine Schiff base 453 with aldehydes 122 (Scheme 84). Similarly, simplified chiral phase-transfer catalyst 457 bearing the 3,5-bis[3,5-bis[4,5]-bi

Scheme 84 Quaternary ammonium salt-catalyzed aldol reactions.

Maruoka suggested that the observed anti-selectivity may be partially attributed to the selective formation of the (E)-enolate. This stereochemistry could be explained by the huge steric repulsion caused by the chiral quaternary ammonium cation, overcoming the gauche interactions between the aldehyde substituent (R<sup>1</sup>) with both the 2-imino moiety and the tert-butoxy group (Figure 20, 458a vs. 458b). On the basis of the product configuration, the re face of the enolate should be shielded effectively by the chiral ammonium cation, and the aldehyde can only approach from the si face.

Figure 20 Proposed transition state models for aldol reactions catalyzed by Maruoka's quaternary ammonium salt.

Corey and coworkers reported a Mukaiyama-type aldol reaction of ketene silyl acetal (Scheme 85–1).  $^{256}$  This reaction, catalyzed by a cinchonidine-derived ammonium bifluoride 461, gave mostly-sm- $\beta$ -hydroxy- $\alpha$ -amino ester 462 as the major diastereomer with good enantiomeric excess. After 6 years, the Andrus group reported catalyst 465 in the aldol reaction between  $\alpha$ -alkoxyacetophenone derivatives 463 and benzaldehyde 464 to give the single sm-product 466 in 76% yield and 80% ee (Scheme 85–2).  $^{257}$ 

#### 2.07.6 Supported Organocatalysis

In general, reaction efficiency, regiochemistry, and stereochemistry can be better predicted in homogeneous catalysis than in heterogeneous catalysis; however, there are limitations to applications of homogeneous systems in the chemical industry.

Scheme 85 Corey's and Andrus' asymmetric aldol reaction.

For example, (1) high-cost chemicals including chiral ligands and noble metals, (2) toxic metal contamination, (3) lability against air, moisture, and heat, and (4) difficulty in catalyst recovering. On the other hand, heterogeneous catalysis is beneficial as the catalysts can be readily separated from the products and reused. Supported organocatalysts are classified as (i) covalently supported catalysts, (ii) noncovalently supported catalysts, and (iii) biphasic or multiphasic systems (Figure 21). Some excellent examples of supported organocatalysts are introduced in this section.

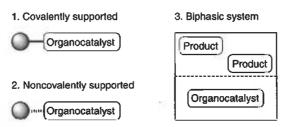


Figure 21 Categories of supported organocatalysts.

### 2.07.6.1 Covalently Supported Organocatalysts

(S)-proline including its derivatives is covalently anchored to an insoluble or soluble support. Polymer and silica are often used as a support due to easy availability, robustness, and wide variety of functional groups. In 1985, the first polystyrene-supported (S)-proline catalyst 467 was reported by the Takemoto group (Figure 22-1).  $^{260}$  Incorporation of a spacer for binding the (S)-proline moiety onto a polymer support is found to improve the enantiomeric excess (n=1, 18% ee; n=7, 39% ee).

Intermolecular aldol reaction in water is performed by the Pericas group, and supported catalyst 468 is prepared by binding reaction of 4-hydroxyproline with a polystyrene resin through click chemistry. The high hydrophobicity of the resin and the presence of water are key to ensuring high stereoselectivity, whereas yield can be increased by using catalytic amounts of water-soluble DiMePEG (Figure 22-2).<sup>261</sup> The soluble poly(ethylene glycol) (PEG)-supported catalyst 469 shows excellent stereoselectivities as similar as that with the nonsupported catalyst reported by the Benaglia and Cozzi group. The soluble PEG-supported catalyst 469 is precipitated, when Et<sub>2</sub>O is added to the reaction mixture. Simple filtration recovers catalyst 469,

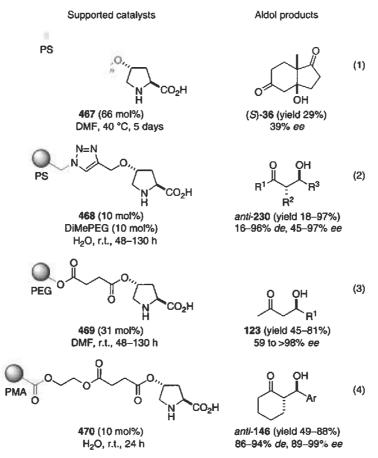


Figure 22 Polymer-supported organocatalyst in aldol reactions.

which is then used for the next reaction (Figure 22-3).<sup>262</sup> A postmodification of polymer beads with catalyst precursors is traditionally employed in the preparation of supported catalysts including organocatalysts. The Hansen group reported an alternative and more scalable approach for polymer-supported organocatalysts. Copolymerization of functional methacrylic monomers affords the desired supported catalyst 470 on multigram scale, which catalyzes the aldol reaction in water to give aldols 146 in excellent yield and enantiomeric excess (Figure 22-4).<sup>263</sup>

Mobile crystalline material 41 (MCM-41), which has a larger specific surface area than other mesoporous silicas, is modified for supported organocatalysts by the Fernández-Mayoralas group (Figure 23). 264,265 MCM-41-supported catalyst 471 provides aldols 158 or 163 in good to excellent yields, even when the reaction is carried out in nonpolar solvent such as toluene, where the use of (S)-proline is hampered by insolubility problems.

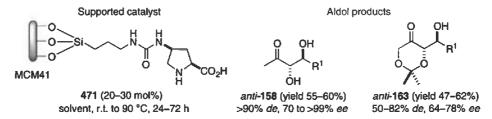


Figure 23 MCM-41-supported organocatalyst in aldol reactions.

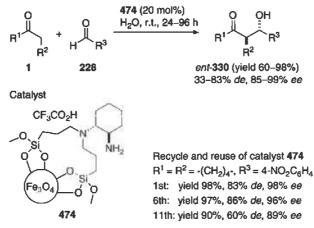
Dendrimers are spheroid or globular nanostructures, the size, shape, and reactivity of which are dependent on generation and chemical composition of the core, interior branching, and surface functionalities.

(S)-proline catalyst supported on surface-functionalized diaminobutane poly(propyleneimine) dendrimers DAB(AM)<sub>8</sub> is evaluated as catalysts for asymmetric aldol reactions reported by the Kokotos group (Figure 24). <sup>266</sup> Using 6.5 mol% of the second

generation modified dendrimer catalyst 472, aldols 123 are obtained in good yield and *ee* comparable to those observed using (*S*)-proline itself. Reaction time with catalyst 472 is shorter than that with (*S*)-proline, as the reaction occurs in complete homogeneous solution due to the increased solubility of dendrimer catalyst 472 in organic solvents. The Portnoy group described the importance of characteristic features to the dendritic architecture on asymmetric aldol reaction (Figure 24).<sup>267</sup> In the aldol reaction with catalyst 473, the proximity of the two proline units is crucial for achieving higher yield and enantioselectivity.

Figure 24 Dendrimer-supported organocatalyst in aldol reactions.

Although nanoscale catalyst recovery is difficult with conventional filtration, very small particles of the supported catalyst would be advantageous because of a large surface-to-volume ratio relative to bulk materials. Recently, the isolation and separation of nanosize catalysts have been achieved using magnetically separable nanoparticles. <sup>268,269</sup> Magnetic nanoparticle-supported chiral primary amine 474 efficiently and stereoselectively catalyzed aldol reactions under on-water conditions. Catalyst 474 can be recycled magnetically and reused up to 11 times with no significant loss of activity or stereoselectivity (Scheme 86). <sup>270</sup>



Scheme 86 Magnetically supported amine-catalyzed aldol reactions.

# 2.07.6.2 Noncovalently Supported Organocatalysts

Organocatalysts are often noncovalently anchored to an insoluble support such as montmorillonite and  $\beta$ -cyclodextrin. Although bond strength in noncovalently supported catalyst is weak, the catalyst itself is directly used for immobilization without need for

modification or synthetic steps required for covalent attachment to support. Inclusion of (S)-proline derivatives with  $\beta$ -cyclodextrin is a useful method for noncovalently supported organocatalyst (Figure 25). The Zhang group demonstrated that the  $\beta$ -cyclodextrin-immobilized catalyst 475 is prepared conveniently by simply heating (4S)-phenoxy-(S)-proline and  $\beta$ -cyclodextrin in ethanol water and by removal of the solvent. The catalyst 475 proceeds in good yields and stereoselectivities, and the catalyst 475 can be recycled four times without loss of enantioselectivity. (4S)-tert-Butylphenoxy-(S)-proline binding a sulfated  $\beta$ -cyclodextrin 477 catalyzes the aldol reaction in water reported by the Armstrong group. The Enantio- and diastereoselectivities up to >99% are achieved for stoichiometric amounts of cyclohexanone and arylaldehydes with this system.

Cyclodextrin-supported catalyst Aldol products

O OH

475 (10 mol%, 
$$\beta$$
-CD,  $R^1$  = Ph)

25 °C, 16–72 h

476 (yield 77–90%)

71–83% ee

O OH

477 (2 mol%, sulfated  $\beta$ -CD,

 $R^1$  = 4- $f$ -BuC<sub>6</sub>H<sub>4</sub>), H<sub>2</sub>O, r.t., 48 h

anti-146 (yield 62–100%)

68 to >98% de, 96 to >99% ee

Figure 25 β-Cyclodextrin-supported organocatalysts.

Polyoxometalates (POMs) are transition metal oxygen clusters with well-defined atomic coordination structures. POMs are used as functional nanocolloidal materials and as supports for catalysts through ion-pair interactions due to their acidic properties. Combinations of chiral diamines and POM 478 effectively catalyze enamine-based aldol reactions. Less than 1 mol% of chiral amine loading is sufficient to catalyze the reaction (Table 7, entries 1 and 2).<sup>274</sup> Highly diastereo- and enantioselective cross-aldol reactions of aldehydes are accomplished using chiral diamine-POM 479 under emulsion condition (entries 3 and 4).<sup>275</sup>

Table 7 Polyoxometalate-supported diamine-catalyzed aldol reactions

Entries	R1	$R^2$	$R^3$	Catalyst (mol%)	Conditions	Yield (%)	dr (anti/syn)	ee (%) <sup>3</sup>	References
1	-(CH <sub>2</sub> ) <sub>4</sub> -		4-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	478 (0.33)	r.t., 16 h	99	87:13	99	274
2	Me	Me	4-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	478 (0.33)	r.t., 19 h	59	90:10	98	274
3	Н	Me	4-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	479 (2.5)	H <sub>2</sub> O, 0 °C, 24 h	92	>95:<5	99	275
4	Н	Me	Ph	479 (2.5)	H <sub>2</sub> O, 0 °C, 80 h	80	>95:<5	99	275
5	-(CH <sub>2</sub> ) <sub>4</sub>		4-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	<b>480</b> (10)	CH <sub>2</sub> Cl <sub>2</sub> , r.t., 16 h	97	91:9	97 <sup>6</sup>	276
6°	-(CH <sub>2</sub> ) <sub>4</sub> -		4-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	<b>480</b> (10)	CH <sub>2</sub> Cl <sub>2</sub> , r.t., 34 h	95	92:8	96 <sup>b</sup>	276

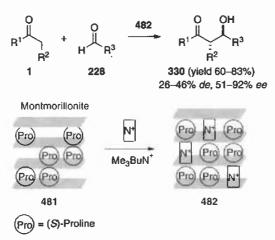
<sup>\*</sup>Enantiomeric excess for the anti-diastereomer.

Enantiomer of 330 was obtained as a major product.

<sup>&</sup>quot;After lour cycles.

Sulfonated polystyrene or fluoropolymer nafion® NR50 is also a good support for the immobilization of primary diamines. The catalyst 480 can be recovered by filtration and reused for at least four cycles with no loss of stereoselectivity (entries 5 and 6). <sup>276,277</sup>

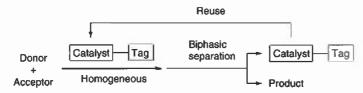
Clays are abundant and available substances that promote a variety of chemical reactions. The characteristic layered structure of clays attracted the attention of synthetic chemists who have used it as a support material. <sup>278</sup> For example, chiral organic-inorganic hybrid materials 482 based on (S)-proline-exchanged clay such as montmorillonite are used for heterogeneous aldol reactions (Scheme 87). Coinclusion of ammonium cations within the layers enhances yields and stereoselectivities. Upon recycling catalyst 482 showed no decrease in enantioselectivity or conversion after 10 cycles. <sup>279</sup>



Scheme 87 Clay-supported (S)-proline-catalyzed aldol reactions.

#### 2.07.6.3 Supported Organocatalysts in Multiphasic Systems

Despite the advantage of easy separation in solid-phase synthesis, the use of insoluble solid-supported catalysts sometimes requires long reaction times, low chemical yields, and low stereoselectivity. Liquid-phase syntheses using soluble linear polymers as catalyst supports have been developed to improve the chemical reactivities. <sup>280</sup> In addition, ionic or fluorous tags have been employed in organocatalytic transformations in recent years (Scheme 88). <sup>281–287</sup>



Scheme 88 Tagged organocatalysts.

Polar organocatalysts such as amino acids and peptides are almost insoluble in conventional organic solvents, but they are soluble in ionic liquids. Owing to these physical properties, asymmetric syntheses in ionic liquids under biphasic condition have been reported. 288,289 The Toma group reported that (S)-proline-catalyzed direct aldol reaction of acetone 113 with arylaldehyde 122 in ionic liquid such as 1- butyl-3-methylimidazolium hexafluorophosphate (Scheme 89). Good yields with reasonable enantioselectivities are achieved, even when just 1-5% of (S)-proline is used. Immobilization of the catalyst in an ionic liquid phase offers simple product isolation, that is, product 123 could be extracted using an immiscible solvent against ionic liquid, and reuse of the catalytic system in subsequent reactions.

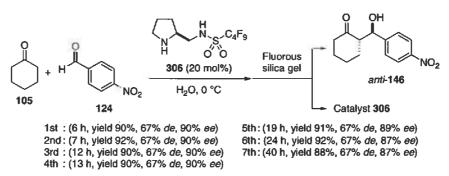
The introduction of an imidazolium tag through acetate connection at the C-4 position of *cis*-4-hydroxy-l-proline **483** provides a highly efficient catalyst for the direct asymmetric aldol reaction that works in a remarkably low catalyst loading (0.1 mol%) with *ee* up to >99% (Scheme 90).<sup>291</sup>

Similar to biphasic system with ionic liquid, fluorous system is also a powerful methodology in organic synthesis. Catalyst 306, fluorous (S)-pyrrolidine sulfonamide, is shown to be a very effective catalyst in the direct aldol reaction in water providing aldol 146 in good yields with high diastereo- and enantioselectivities as reported by the Wang group (Scheme 91). 155 Fluorous separation allows catalyst recovery and reuse for seven cycles without a significant loss of catalytic activity and stereoselectivity.

#### 334 The Aldol Reaction: Organocatalysis Approach

**Scheme 89** Reuse of (S)-proline in aldol reaction in ionic liquids.

Scheme 90 Aidol reaction using ionic tag.



Scheme 91 Recovery of the fluorous catalyst.

Recently, combinations of solid catalysts and ionic liquids have been intensively studied. The supported ionic liquid phase catalyst is a new generation of the supported liquid-phase catalyst.<sup>292</sup>

Polar molecules such as (S)-proline are absorbed onto the monolayer surface of silica gel covalently attached to ionic liquid with additional ionic liquid reported by the Gruttadauria group (Figure 26). <sup>293</sup> These layers serve as the reaction phase as well as the supported homogeneous catalyst phase. Good yields and enantiomeric excess comparable to those obtained with (S)-proline itself are observed; in addition, the supported catalyst 484 shows high reusability. Polar ionic-liquid-supported catalysts are precipitated through biphasic separation by adding a less polar solvent into the reaction mixture, and the recovered catalyst can be reused. Polyelectrolytes are also a good support for (S)-proline. The (S)-proline/poly(diallyldimethylammonium) hexafluorophosphate combination as a supported catalyst 485 is developed by the Zlotin group. <sup>294</sup> Recycling of catalyst 485 is possible at least six times without loss of activity and enantioselectivity in asymmetric aldol reaction.

The supported ionic liquid phase catalyst 486 is readily prepared by adsorption of (S)-proline 6 or peptide 487 onto the surface of silica gels covalently functionalized with an ionic monolayer. These supported catalysts catalyze the aldol reaction between

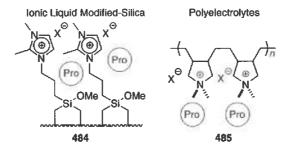


Figure 26 Proline catalyst in multiphase.

acetone 113 and aldehyde 124 to give the desired product 123a in high yields. The supported catalyst 486 is easily recovered by filtration and can be used several times without loss of either conversion or selectivity (Scheme 92).<sup>295</sup>

Scheme 92 Supported ionic liquid phase catalyst.

# 2.07.7 Conclusions

The past two decades have been an age of exploration and discovery in organocatalysis, and new and powerful synthetic tools have been developed. The catalytic asymmetric assembly of readily available precursor molecules gives rise to complex products with extremely high stereoselectivities under operationally simple and, in some cases, environmentally friendly experimental conditions. Until now, organocatalysis has been developing at a remarkable pace, but there is still room for improvement, as the generality scope is not yet determined; diastereoselectivities and enantioselectivities are still low in some cases, and high catalyst loadings are still required. It is believed that this rapidly growing field will rise to meet these challenges in the near future.

For related chapters in this Comprehensive, you can refer to - Chapters 2 11, 4.03, 2.08, 2.09, 2.10, 2.13, and 2.12.

## References

- 1. Kane, R J. Prakt Chemie 1838, 15, 129-155
- 2. Wurtz, A. J. Prakt. Chemie 1872, 5, 457-464
- 3. Mukaiyama, T. Aldrichimica Acta 1996, 29, 59-76
- Hajos, Z. G., Parrish, D. R. (Hoffmann-La Roche, F., und Co., A.-G.) Asymmetric Synthesis of Optically Active Polycyclic Organic Compounds. German Pat. Appl. 2,102.
   623, 1971.
- 5. Hajos, Z. G., Parrish D R J. Org Chem 1974 39 1615-1621
- 6 Eder, U., Wiechert, R.; Sauer, G. (Schering A -G.) Optically Active 1.5-Indandione and 1.6-Naphthalenedione Derivatives German Pat. Appl. 2,014.757, 1971.
- 7 Eder U; Sauer G; Wiechert, R Angew Chem. Int Ed 1971 10 496-497
- 8. Yamada, Y. M. A.; Yoshikawa, N.; Sasai, H.; Shibasaki, M. Angew. Chem. Int. Ed. 1997, 36, 1871-1873.
- 9 List 8.; Lerner, R A., Barbas, C F., III J Am Chem Soc 2000 122, 2395-2396

#### 336 The Aldol Reaction: Organocatalysis Approach

```
10. Barbas, C. F., Ili. Angew. Chem. Int. Ed. 2008, 47, 42-47.
 11. MacMillan, D. W. C. Nature 2008, 455, 304-308.
 12. Bertelsen, S., Jørgensen, K. A. Chem. Soc. Rev. 2009, 38, 2178-2189
 13. Melchiorre, P., Marigo, M.; Carlone, A.; Bartoli, G. Angew Chem. Int. Ed. 2008. 47 6138-6171
 14. Dondoni, A.; Massi, A. Angew. Chem. Int. Ed. 2008, 47, 4638-4660.
 15. Gaunt, M. J., Johansson, C. C. C.; McNally, A., Vo, N. T. Drug Discov. Today 2007, 12, 8-27.

    Jaroch, S.; Weinmann, H.; Zeitler, K. ChemMedChem 2007, 2, 1261–1264.

 17. Pellissier, H. Tetrahedron 2007, 63, 9267-9331
 18. Trost, B M.; Brindle, C S Chem Soc Rev 2010, 39, 1600-1632
 19. Adachi, S.; Harada, T. Eur. J. Org. Chem. 2009, 3661-3671
 20. Geary, L. M.; Hultin, P. G. Tetrahedron Asymmetry 2009, 20, 131-173.
 21. Palomo, C.; Giarbide, M., Laso, A. Eur. J. Org. Chem. 2007, 2561-2574.

    Zlotin, S. G.; Kucherenko, A. S., Beletskaya, I. P. Russ. Chem. Rev. 2009, 78, 737–784.
    Bhanushali, M., Zhao, C.-G. Synthesis 2011, 1815–1830

 24. Guillena, G.; Nájera, C.; Ramón, D. J. Tetrahedron Asymmetry 2007, 18, 2249-2293
 25. Dean, S. M.; Greenberg, W. A.; Wong, C.-H. Adv. Synth. Catal. 2007, 349, 1308-1320.
 26. Machajewski, T. D.; Wong, C.-H. Angew Chem Int. Ed. 2000, 39, 1352-1375
 27. Whalen, L. J.; Wong, C.-H. Aldrichim. Acta 2006, 39, 63-71
 28. Wagner, J.; Lerner, R. A.; Barbas, C. F.; III. Science 1995, 270, 1797-1800.
 29. Barbas, C. F., III; Heine, A.; Zhong, G.; et al. Science 1997, 278 2085-2092
 30. Mukherjee, S.; Yang, J. W., Hoffmann, S.; List, B. Chem. Rev. 2007, 107, 5471-5569.
31. Moyano, A.; Rios, R. Chem. Rev. 2011, 111, 4703-4832.
32. Nagamine, T.; Inomata, K.; Endo, Y., Paquette, L. A. J. Org. Chem. 2007, 72, 123-131
33. Davies, S. G., Sheppard, R. L., Smith, A. D.; Thomson, J. E. Chem. Commun. 2005, 3802-3804
34. Limbach, M. Tetrahedron Lett. 2006, 47, 3843-3847
35. But, T., Barbas, C. F., III. Tetrahedron Lett. 2000, 41, 6951-6954
36. Danishefsky, S. J.: Masters, J. J., Young, W. B.; et al. J. Am. Chem. Soc. 1996, 118, 2843-2859
37. Agami, C.; Sevestre, H. J. Chem. Soc., Chem. Commun. 1984, 1385-1386
38. Zhou, J.; Wakchaure, V., Kraft, P.; List, B. Angew. Chem. Int. Ed. 2008, 47, 7656-7658
39. Enders, D.: Niemeier, O; Straver, L. Synlett 2006, 3399-3402
 40. Chandler, C L., List, B J Am. Chem Soc 2008, 130, 6737-6739.
41. Woodward, R. B.; Logusch, E.; Nambrar, K. P.; et al. J. Am. Chem. Soc. 1981, 103, 3210-3213
42. Agami, C.; Platzer, N.; Puchot, C.; Sevestre, H. Tetrahedron 1987, 43 1091-1098.
43. Itagaki, N.; Kimura, M.; Sugahara, T.; Iwabuchi, Y. Org. Lett. 2005, 7, 4185-4188.
44. flagaki, N.; Sugahara, T.; Iwabuchi, Y. Org. Lett. 2005, 7, 4181-4183
45. Itagaki, N., Iwabuchi, Y. Chem. Commun. 2007, 1175-1176
46. Diaba, F.; Bonjoch, J. Org. Biomot. Chem 2009, 7, 2517-2519.

    Prdathala, C., Hoang, L., Vignola, N., List, B. Angew. Chem. Int. Ed. 2003, 42, 2785–2788
    Yoshitomi, Y., Makino, K., Harnada, Y. Org. Lett. 2007, 9, 2457–2460

49. Hayashi, Y.; Sekizawa, H.; Yamaguchi, J., Gotoh, H. J. Org. Chem. 2007, 72, 6493-6499
50. Mans. D. M.; Pearson, W. H. Org. Lett. 2004, 6, 3305-3308.
51. Tokuda, O.; Kano, T.; Gao, W.-G., Ikemoto, T., Maruoka, K. Org. Lett. 2005, 7, 5103-5105.
52. Samanta, S.; Zhao, C.-G. Tetrahedron Lett. 2006, 47, 3383-3386.
53. Samanta, S.; Zhao, C.-G. J. Am. Chem. Soc. 2006, 128, 7442-7443
54. Liu, J.; Yang, Z.; Wang, Z.; et al. J. Am. Chem. Soc. 2008, 130, 5654-5655.

    Wang, Y., Shen, Z.; Li, B.; Zhang, Y.; Zhang, Y. Chem Commun. 2007, 1284–1286
    Tang, Z.; Cun, L.-F.; Cui, X.; et al. Org. Lett. 2006, 8 1263–1266

57. Saklhivel, K.; Notz, W.; Bui, T.; Barbas, C. F. III. J. Am. Chem. Soc. 2001, 123, 5260-5267.
58. Hayashi, Y.; Yamaguchi, J., Hibino, K., et al. Adv. Synth. Catal. 2004, 346, 1435-1439.
59. Hayashi, Y., Sumiya, T.; Takahashi, J.; et al. Angew. Chem. Int. Ed. 2006, 45, 958-961.
60. Aratake, S., Iloh, T.; Okano, T., et al. Chem. Eur. J. 2007, 13, 10246-10256.
61. Liu, X; Lin, L; Feng, X Chem. Commun 2009, 6145-6158
62. Tang, Z.; Yang, Z.-H.; Chen, X.-H.; et al. J. Am. Chem. Soc. 2005, 127, 9285-9289.
63. Yang, H., Carter, R. G. Synlett 2010, 2827-2838
64. Berkessel, A.; Koch, B., Lex, J. Adv. Synth. Catal. 2004, 346, 1141-1146.
65. Saito, S.; Yamamoto, H. Acc. Chem. Res. 2004, 37, 570-579
66. Mase, N; Tanaka, F.; Barbas, C. F., III. Angew Chem Int Ed 2004, 43, 2420-2423
67. Longbottom, D. A.; Franckevičius, V.; Kumarn, S., et al. Aldrichimica Acta 2008, 41, 3-11
68. Torii, H.; Nakadai, M.; Ishihara, K.; Saito, S.; Yamamoto, H. Angew. Chem. Int. Ed. 2004, 43, 1983-1986
69. Cobb, A J A.; Shaw, D M.; Ley, S V Synlett 2004, 558-560.
70. Hartikka, A.; Arvidsson, P. I. Tetrahedron Asymmetry 2004, 15, 1831-1834
71. Hayashi, Y. Itoh, T.; Aratake, S.; Ishikawa, H. Angew Chem Int. Ed. 2008, 47. 2082-2084
72. Hayashi, Y., Samanta, S., Itoh, T.; Ishikawa, H. Org. Lett. 2008, 10, 5581-5583
73. Itoh, T.; Ishikawa, H.; Hayashi, Y. Org. Lett. 2009, 11, 3854-3857
74. Urushima, T.; Yasur, Y.; Ishikawa, H.; Hayashi, Y. Org. Lett. 2010, 12, 2966-2969.
75. Davie, E. A. C.; Mennen, S. M.; Xu, Y.; Miller, S. J. Chem. Rev. 2007, 107, 5759-5812
76. Martin H J, List, B. Synlett 2003, 1901-1902
77. Krattiger, P.; Kovasy, R.; Revell, J. D.; Ivan, S.; Wennemers, H. Org. Lett. 2005, 7, 1101-1103
78. Gu. Q., Wang, X.-F.; Wang, L., Wu, X.-Y.; Zhou, Q.-L. Tetrahedron Asymmetry 2006, 17, 1537-1540.
79. Liu, X-W; Le, T. N., Lu, Y.; et al. Org. Biomol. Chem. 2008, 6, 3997-4003
80 Tang, X; Liégault B; Renaud, J-L; Bruneau, C. Tetrahedron Asymmetry 2006, 17, 2187-2190.
81. Kano, T., Takai, J.; Tokuda, O.; Maruoka, K. Angew Chem. Int. Ed. 2005, 44, 3055-3057.
82. Kano, T.; Tokuda, O.; Takai, J.; Maruoka, K. Chem. Asian J. 2006. 1, 210-215.
```

```
83 Kano, T., Tokuda, O., Maruoka, K. Tetrahedron Lett. 2006, 47 7423-7426
 84 List, B : Pojarliev, P. Castello, C. Org. Lett. 2001, 3, 573-575
 85 Notz, W., List, B. J. Am. Chem. Soc. 2000, 122, 7386-7387
 86 Enders, D ; Grondal, C. Angew Chem Int. Ed. 2005, 44, 1210-1212
 87. Suri, J. T., Ramachary, D. B., Barbas, C. F. III. Org. Lett. 2005. 7, 1383-1385.
 88. Sekiguchi, Y.; Sasaoka, A.; Shimomoto, A.; Fujioka, S.; Kotsuki, H. Synlett 2003, 1655-1658.
 89 Loh, T.-P., Feng, L.-C., Yang, H.-Y., Yang, J.-Y. Tetrahedron Lett. 2002 43, 8741-8743
 90 North, M.; Villuendas, P. Org. Lett. 2010, 12, 2378-2381.
 91 Chan V; Kim, J. G.; Jimeno, C., Carroll, P. J.; Walsh, P. J. Org. Lett. 2004. 6, 2051-2053.
 92. Chowdari, N. S., Ramachary, D. B.; Barbas, C. F., III. Org. Lett. 2003, 5, 1685-1688.
 93. Zheng, Y.; Avery, M. A. Tetrahedron 2004, 60, 2091-2095
 94. Doi, T.: Numajiri, Y.; Munakata, A., Takahashi, T. Org. Lett. 2006. 8, 531-534.
 95 Zou, B., Wei, J.; Cai, G.; Ma, D. Org. Lett. 2003, 5, 3503-3506.
 96 Reymond J.-L., Chen, Y. J. Org. Chem. 1995, 60, 6970-6979
 97. Bahmanyar, S., Houk, K. N. J Am Chem Soc 2001, 123, 11273-11283
 98. Xu, L.-W; Luo, J.; Lu, Y. Chem. Commun. 2009, 1807-1821
 99 Xu, L-W, Lu, Y Org Biomol Chem 2008 6, 2047-2053
100. Peng, F.; Shao, Z. J. Mol. Catal. A. Chem. 2008, 285, 1-13
101. Chen, Y -C Synlett 2008, 1919-1930
102. Córdova, A., Zou, W. Oziedzic, P. et al. Chem. Eur. J. 2006, 12, 5383-5397
103. Hayashi, Y.; Itoh, T.; Nagae, N.; Ohkubo, M., Ishikawa, H. Synlett 2008, 1565-1570
104. Tanaka, F.; Thayumanavan, R., Mase, N., Barbas, C. F., III. Tetrahedron Lett. 2004, 45, 325-328
105. Jiang, Z.; Yang, H.; Han, X.; et al. Org. Biomol. Chem. 2010, 8, 1368-1377
106. Bassan, A.; Zou, W.; Reyes, E.; Himo, F.; Córdova, A. Angew Chem Int. Ed. 2005. 44, 7028-7032.
107. Nakayama, K.; Maruoka, K. J. Am. Chem. Soc. 2008. 130, 17666-17667
108. Kobayashi, S. Pure Appl. Chem. 2007, 79, 235-245
109 Casas J. Sundén H., Cordova, A Tetrahedron Lett 2004, 45, 6117-6119
110. Mase, N.; Inoue, A.; Nishio, M.; Takabe, K. Bioorg. Med. Chem. Lett. 2009, 19. 3955-3958
111. Ramasastry, S. S. V.; Zhang, H.; Tanaka, F., Barbas, C. F. III. J. Am. Chem. Soc. 2007, 129, 288-289.
112. Wu, X.; Jiang, Z.; Shen, H.-M.; Lu, Y. Adv. Synth. Catal. 2007. 349, 812-816.
113. Ramasastry, S. S. V., Albertshofer, K., Utsumi, N., Tanaka, F.; Barbas, C. F., III. Angew. Chem. Int. Ed. 2007. 46, 5572–5575.
114. Utsumi, N., Imai, M.; Tanaka, F.; Ramasastry, S. S. V.; Barbas, C. F., III. Org. Lett. 2007, 9, 3445-3448.
115. Córdova, A., Notz, W.; Barbas, C. F., III. Chem. Commun. 2002. 3024-3025
116 Lup, S. Xu, H. Li, J.; Zhang, E., Cheng, J.-P. J. Am. Chem. Soc. 2007, 129, 3074-3075.
117. Bøgevig, A. Kumaragurubaran, N., Jørgensen, K. A. Chem. Commun. 2002. 620-621
118. Córdova, A.; Notz, W.; Barbas, C. F., III. J. Org. Chem. 2002, 67, 301-303
119. Chowdari, N. S.; Ramachary, D. B.; Cordova, A., Barbas, C. F., Ili. Tetrahedron Lett. 2002, 43 9591-9595
120. Northrup, A. B., MacMillan, D. W. C. J. Am. Chem. Soc. 2002, 124, 6798-6799
121. Northrup, A. B.; Mangion, I. K.; Hettche, F.; MacMillan, D. W. C. Angew Chem. Int. Ed. 2004, 43, 2152-2154.
122. Northrup, A. B.; MacMillan, D. W. C. Science 2004, 305, 1752-1755
123. Córdova, A.; Ibrahem, I.; Casas, J., et al. Chem. Eur. J. 2005, 11, 4772-4784.
124. Thayumanavan, R.; Tanaka, F., Barbas, C. F., III. Org. Lett. 2004, 6, 3541-3544
125. Mangion, I. K.; Northrup, A. B., MacMillan, D. W. C. Angew Chem. Int. Ed. 2004, 43, 6722-6724.
126. Kano, T.; Yamaguchi, Y.; Tanaka, Y., Maruoka, K. Angew Chem. Int. Ed. 2007, 46, 1738-1740.
127. Kano, T.: Sugimoto, H., Maruoka K. J. Am. Chem. Soc 2011, 133, 18130-18133
128. Hayashi, Y.; Yasui, Y.; Kawamura, T.; Kojima, M.; Ishikawa, H. Angew. Chem. Int. Ed. 2011, 50, 2804-2807
129. Hayashi, Y.; Yasui, Y.; Kojima, M.; Kawamura, T.; Ishikawa, H. Chem. Commun. 2012. 48, 4570-4572.
130. Hayashi, Y.; Yasur, Y.; Kawamura, T.; Kojima, M., Ishikawa, H. Synlett 2011, 485-488
131. Mangion, I. K.; MacMillan, D. W. C. J. Am. Chem. Soc. 2005, 127, 3696-3697
132. Hayashi, Y. Shoji, M., Ishikawa, H.; et al. Angew Chem. Int. Ed. 2008, 47, 6657-6660
133. Butler, R N; Coyne, A G Chem Rev 2010, 110, 6302-6337
134. Paradowska, J.; Stodulski, M.; Mlynarski, J. Angew. Chem. Int. Ed. 2009, 48, 4288-4297
135. Pana, C., Wang, Z. Coord Chem Rev 2008, 252, 736-750
136. Rideout, D. C., Brestow, R. J. Am. Chem. Soc. 1980, 102, 7816-7817
137. Breslow, R. Acc Chem Res 1991 24, 159-164.
138 Narayan, S. Muldoon, J.; Finn, M. G.; et al. Angew. Chem. Int. Ed. 2005, 44 3275-3279
139 Mlynarski, J.; Paradowska, J. Chem. Soc. Rev. 2008, 37, 1502-1511
140, Mase N.; Barbas, C F III Org. Biomol. Chem 2010, 8, 4043-4050
141. Gruttadauria, M., Gracalone, F., Noto, R. Adv. Synth. Catal. 2009, 351, 33-57.
142. Raj, M.; Singh, V K Chem Commun 2009, 6687-6703
143. Nyberg, A. I.; Usano, A., Pihko, P. M. Synlett 2004, 1891-1896
144. Amedikouh, M. Tetrahedron Asymmetry 2005 16, 1411-1414
145. Córdova A., Zou, W., Ibrahem, I.; et al. Chem. Commun. 2005, 3586-3588
146 Zou, W., Ibrahem, I.; Dziedzic, P.; Sundén, H., Córdova, A. Chem. Commun. 2005, 4946-4948
147. Mase, N.; Nakai, Y., Ohara, N., et al. J. Am. Chem. Soc. 2006, 128, 734-735
148. Mase, N.; Noshiro, N.; Mokuya, A.; Takabe, K. Adv. Synth. Catal. 2009. 357, 2791-2796
149. Hayashi, Y. Angew Chem. Int. Ed 2006 45, 8103-8104.
150. Brogan, A. P., Dickerson, T. J., Janda, K. D. Angew Chem Int. Ed. 2006, 45, 8100-8102
151. Blackmond, D. G., Armstrong, A.; Coombe, V.; Wells, A. Angew Chem. Int. Ed. 2007. 46, 3798-3800.
152. Gryko, D., Saletra, W. J. Org. Biomol. Chem. 2007, 5, 2148-2153
153. Maya V; Raj, M; Singh, V; K. Org. Lett 2007, 9, 2593—2595
154. Zhao, J.-F., He, L.; Jiang, J.; et al. Tetrahedron Lett. 2008, 49, 3372—3375.
155. Zu, L.; Xie, H.; Li, H.; Wang, J.; Wang, W. Org. Lett. 2008, 10, 1211-1214.
```

#### 338 The Aldol Reaction: Organocatalysis Approach

```
156. Lin. J.-H.: Zhang, C.-P.: Xiao, J.-C. Green Chem. 2009, 11, 1750-1753
 157. Hayashi, Y., Aratake, S.; Okano, T., et al. Angew. Chem. Int. Ed. 2006, 45, 5527-5529
 158. Zhong, L., Gao, Q., Gao, J., Xiao, J.; Li, C. J. Catal. 2007, 250, 360-364
 159. Jung, Y; Marcus, R. A. J. Am. Chem. Soc. 2007, 129, 5492-5502
 160 Pirrung, M. C. Chem Eur J. 2006, 12, 1312-1317.
 161. Aratake, S., Itoh, T.; Okano, T., et al. Chem. Commun. 2007. 2524-2526.
 162. Lindström, U. M., Andersson, F. Angew Chem. Int. Ed. 2006, 45, 548-551
 163. Gruttadauria, M., Gracalone, F.; Marculescu, A. M., et al. Eur. J. Org. Chem. 2007, 4688-4698
 164. Shao, Z.: Zhang, H. Chem. Soc. Rev. 2009, 38, 2745-2755.
 165. Ambrosini, L. M.; Lambert, T. H. ChemCatChem 2010, 2, 1373-1380
 166. Zhong, C., Shi, X. Eur. J. Org. Chem. 2010, 2999-3025.
 167. Zhou, J. Chem. Asian J. 2010, 5, 422-434.
 168 Abillard, O.; Breit, B. Adv. Synth. Catal. 2007, 349, 1891-1895
 169 Edin, M.; Bäckvall, J.-E.; Córdova, A. Tetrahedron Lett. 2004, 45, 7697-7701
 170 Baer, K., Kraußer, M., Burda, E., et al. Angew. Chem. Int. Ed. 2009, 48, 9355-9358.
171. Seebach, D. Beck, A. K., Badine, D. M.; et al. Helv. Chim. Acta 2007, 90, 425-471.
172. Zolova, N.; Broadbelt, L. J.; Armstrong, A.; Blackmond, D. G. Bioorg, Med. Chem. Lett. 2009, 19, 3934-3937.
173. Wieland, P; Miescher, K. Helv. Chim Acta 1950 33, 2215-2228.
174. Spencer, T. A.; Neel, H. S.; Flechtner, Y. W., Zayle, R. A. Tetrahedron Lett. 1965, 6, 3889–3897.
175. List, B.; Hoang, L., Martin, H. J. Proc. Natl. Acad. Sci. USA 2004, 101, 5839-5842.
176. Puchot, C., Samuel, O.; Dunach, E., et al. J. Am. Chem. Soc. 1986, 108, 2353-2357
177. Agami, C., Levisaffes, J.; Puchot, C. J. Chem. Soc., Chem. Commun. 1985, 441-442
178. Cheong, P. H.-Y. Legault, C. Y.: Um, J. M. Celebi-Olcum, N., Houk, K. N. Chem. Rev. 2011, 111, 5042-5137
179 Bahmanyar, S.; Houk, K. N. J. Am. Chem. Soc. 2001, 123, 12911-12912
180, Hoang, L., Bahmanyar, S., Houk, K. N.; List, B. J. Am. Chem. Soc. 2003, 125, 16-17
181. Bahmanyar, S.; Houk, K. N.; Martin, H. J.; List, B. J. Am. Chem. Soc. 2003, 125, 2475-2479.

    Allemann, C., Gordillo, R.; Clemente, F. R.; Cheong, P. H.-Y.; Houk, K. N. Acc Chem. Res. 2004, 37 558–569
    Akiyama, T., Itoh, J., Fuchibe, K. Adv. Synth. Catal. 2006, 348, 999–1010

184. Doyle. A. G.; Jacobsen, E. N. Chem. Rev. 2007, 107, 5713-5743.
185. Akiyama, T. Chem Rev. 2007, 107, 5744-5758
186. Yu, X; Wang, W Chem Asian J 2008, 3, 516-532
187. Schenker, S., Zamfir, A.; Freund, M.; Tsogoeva, S. B. Eur. J. Org. Chem. 2011, 2209-2222
188. Rueping, M.; Kuenkel, A.; Atodiresei, I. Chem. Soc. Rev. 2011, 40, 4539-4549
189. Yu. J.; Shi, F.; Gang, L.-Z. Acc. Chem. Res. 2011, 44, 1156-1171
190 Adair, G., Mukherjee, S., List, B. Aldrichimica Acta 2008, 41, 31-39
191 Rueping, M., Nachtsheim, B. J., Jeawsuwan, W.; Alodiresei, J. Angew. Chem. Int. Ed. 2011, 50, 6706-6720.
192 Terada, M. Chem. Commun 2008, 4097-4112
193 Zamfir, A.; Schenker, S., Freund, M., Tsogoeva, S. B. Org. Biomol. Chem. 2010. 8 5262-5276.
194 Terada, M Synthesis 2010, 1929-1982.
195 Terada M Bull Chem. Soc. Jpn 2010, 83, 101-119.
196. Zhang, Z. Schreiner P. R. Chem. Soc. Rev. 2009. 38, 1187-1198.
197. Pellissier, H. Tetrahedron 2008, 64, 10279-10317
198. Review of squaramide catalyst Alemán, J., Parra, A.; Jiang, H.; Jørgensen, K. A. Chem. Eur. J. 2011, 17, 6890-6899
199. Terada, M., Tanaka, H.; Sorimachi, K. J. Am. Chem. Soc. 2009, 131, 3430-3431.
200. Zhuang, W., Poulsen, T. B.; Jørgensen, K. A. Org. Biomol. Chem. 2005, 3, 3284-3289
201. McGilvra, J. D.; Unni, A. K.; Modi, K.; Rawal, V. H. Angew Chem. Int. Ed. 2006, 45, 6130-6133
202 Cheon, C. H., Yamamoto, H. Org. Lett. 2010, 12, 2476-2479.
203. García-García, P.; Lay, F.; García-García, P.; Rabalakos, C.; List, B. Angew Chem. Int. Ed. 2009, 48, 4363–4366
204 Huang, Y. Unni, A. K.; Thadani, A. N.; Rawal, V. H. Nature 2003, 424, 146, 146.
205. Gondi, V B.; Hagihara, K., Rawal, V H. Angew Chem Int. Ed 2009, 48, 776-779
206. Casıraghi, G.; Batlıstini, L.; Curli, C.; Rassu, G.; Zanardi, F. Chem. Rev. 2011, 111, 3076-3154.
207. Gondi, V. B.; Gravel, M., Rawal, V. H. Org. Lett. 2005, 7, 5657-5660
208. Pansare, S. V., Paul, E. K. Chem. Eur. J. 2011, 17, 8770-8779.
209. Singh, R. P.; Foxman, B. M.; Deng, L. J. Am. Chem. Soc. 2010, 132, 9558-9560
210. Ratjen, L., Garcia-Garcia, P.; Lay, F.; Beck, M. E., List, B. Angew Chem Int. Ed. 2011, 50, 754-758.
211. Pousse, G., Cavelier, F. L.; Humphreys, L.; Rouden, J.; Blanchet, J. Org. Lett. 2010, 12, 3582-3585.
212. Mori, K.; Katoh, T.; Suzuki, T., et al. Angew. Chem. Int. Ed. 2009, 48, 9652-9654
213. Palomo, C; Oiarbide, M., López, R. Chem. Soc. Rev. 2009, 38, 632-653
214. Leow, D; Tan, C.H. Chem. Asian J. 2009, 4, 488-507
215. Leow, D.; Tan, C.-H. Synlett 2010, 1589-1605
216. Siau, W.-Y.; Wang, J. Catal. Sci. Technol. 2011, 1, 1298-1310.
217. Connon, S. J. Chem. Commun. 2008, 2499-2510.
218. Ube, H., Shimada, N.; Terada, M. Angew Chem. Int. Ed. 2010, 49, 1858-1861.
219. Luo; Wang, H. Han X, et al. Angew. Chem. Int. Ed. 2011, 50, 1861-1864
220. Yang, Y.; Zheng, K.; Zhao, J.; et al. J. Org. Chem. 2010, 75, 5382-5384.
221. Pansare, S. V. Paul, E. K. Chem. Commun. 2011, 47, 1027-1029
222. Misaki, T.; Takimoto, G.; Sugamura, T. J. Am. Chem. Soc. 2010. 132, 6286-6287.
223. Tian, S.-K; Chen, Y; Hang, J.; et al. Acc. Chem. Res. 2004, 37, 621-631
224. Calter, M. A.; Phillips, R. M.; Flaschenriem, C. J. Am. Chem. Soc. 2005, 127, 14566-14567.
225. Ogawa, S., Shibata, N.; Inagaki, J.; et al. Angew. Chem. Int. Ed. 2007, 46, 8666-8669.
226. Guo, Q., Bhanushali, M.: Zhao, C.-G. Angew Chem Int. Ed 2010 49, 9460-9464.
227. Purohit, V. C.; Malla, A. S.; Romo, D. Heterocycles 2008, 76, 949-979
228. Borrmann, D., Wegler, R. Chem. Ber. 1966, 99, 1245-1251
```

```
    Borrmann, D. Wegler, R. Chem. Ber. 1967 100. 1575–1579.
    Borrmann, D.; Wegler, R. Chem. Ber. 1969 102. 64–70

231. Wynberg, H.; Staring, E. G. J. J. Am Chem. Soc. 1982 104, 166-168
232. Wynberg, H.; Staring, E. G. J. J. Org. Chem. 1985, 50, 1977-1979
233. Tennyson R.: Romo D. J. Org. Chem 2000, 65, 7248-7252
234. Cortez, G. S., Tennyson, R. L.; Romo, D. J. Am. Chem. Soc. 2001, 123, 7945-7946
235. Calter. M. A.; Orr. R. K.; Song, W. Org. Lett. 2003, 5, 4745-4748.
236. Mondal, M.; Ibrahim, A. A., Wheeler, K. A.; Kerrigan, N. J. Org. Lett. 2010, 12, 1664-1667
237. Lee, E. C., Hodous, B. L.; Bergin, E., Shih, C.; Fu, G. C. J. Am. Chem. Soc. 2005. 127 11586-11587
238. Curran, D. P., Kuo, L. H. J. Org. Chem. 1994, 59, 3259-3261
239. Sigman, M. S.; Jacobsen, E. N. J. Am. Chem. Soc. 1998, 120, 4901-4902
240. Okino, T. Hoashi Y.: Takemoto, Y. J. Am. Chem. Soc. 2003, 125, 12672-12673
241 Lt, Lt; Klauber, E. G.; Seidel, D. J. Am. Chem. Soc. 2008, 130, 12248-12249
242 Maruoka, K.; Ooi, T Chem Rev 2003 103, 3013-3028
243. Hashimoto, T.; Maruoka, K. Chem. Rev. 2007, 107, 5656-5682.
244, Doi, T.: Maruoka, K. Aldrichimica Acta 2007. 40, 77-86.
245. Ooi, T., Maruoka, K. Angew Chem Int. Ed. 2007, 46, 4222-4266
246 Maruoka, K.; Ooi T.; Kano, T Chem Commun 2007 1487-1495
247. Jew S.-s; Park, H.-g. Chem. Commun. 2009, 7090-7103
248. Maruoka, K. Org. Process Res. Dev. 2008, 12, 679-697
249. Dolling, U. H., Davis, P.; Grabowski, E. J. J. J. Am. Chem. Soc. 1984, 106, 446-447
250. Gasparski, C. M.; Miller, M. J. Tetrahedron 1991, 47, 5367-5378
251. Mettath, S., Srikanth, G. S. C., Dangerfield, B. S.; Casile, S. L. J. Org. Chem. 2004, 69, 6489-6492.
252. Goi, T.; Taniguchi, M.; Kameda, M.; Maruoka, K. Angew Chem Int. Ed. 2002, 41, 4542-4544.
253. Ooi, T., Kameda, M.; Taniguchi, M.; Maruoka, K. J. Am. Chem. Soc. 2004, 126, 9685-9694.
254. Kano, T.; Lan, Q.; Wang, X.; Maruoka, K. Adv. Synth. Catal. 2007, 349, 556-560.
255. Kitamura, M.; Shirakawa, S.; Arimura, Y.; Wang, X.; Maruoka, K. Chem. Asian J. 2008. 3, 1702-1714.
256. Horikawa, M.; Busch-Petersen, J.; Corey, E. J. Tetrahedron Lett. 1999, 40: 3843-3846
257 Andrus M. B.; Liu J., Ye, Z.; Cannon J F Org Lett 2005, 7, 3861-3864
258. Gruttadauna, M., Giacalone, F.; Noto, R. Chem. Soc. Rev. 2008. 37, 1666-1688
259. Kristensen, I. E.; Hansen, I. Eur. J. Urg. Chem. 2010. 3179-3204
260. Kondo, K.; Yamano, T., Takemoto, K. Makromol, Chem. 1985, 186, 1781-1785.
261 Font, D.; Jimeno, C.; Pericas, M. A. Org. Lett. 2006 8 4653-4655
262. Benaglia, M.; Celentano, G.: Cozzi, F. Adv. Synth. Catal. 2001, 343, 171-173.
263 Kristensen, T. E.; Vestli, K.; Jakobsen, M. G.; Hansen, F. K.; Hansen, T. J. Org. Chem. 2010, 75, 1620-1629.
264. Calderon, F.; Fernández, R.; Sánchez, F.; Fernández-Mayoralas, A. Adv. Synth. Calal. 2005, 347, 1395-1403.
265. Doyaguez, E. G.; Calderón, F., Sánchez, F.; Fernández-Mayoralas, A. J. Org. Chem. 2007, 72, 9353-9356.
266. Bellis, E.: Kokotos, G. J. Mol. Calal. A: Chem. 2005, 241, 166-174
267. Goren, K.; Kehal, T.; Portnoy, M. Adv. Synth. Catal. 2009. 351, 59-65.
268. Polshetliwar, V; Luque, R, Fihri, A; et al. Chem. Rev. 2011. 111. 3036-3075
269. Shylesh, S., Schünemann, V.; Thiel, W. R. Angew. Chem. Int. Ed. 2010, 49, 3428-3459

    Luo, S.; Zheng, X.; Cheng, J.-P. Chem Commun 2008, 5719–5721
    Zhang, L.; Luo, S.; Cheng, J.-P. Catal. Sci. Technol. 2011, 1, 507–516

272. Shen, Z.; Ma, J.; Liu, Y.; et al. Chirality 2005. 17, 556-558
273. Huang, J.; Zhang, X., Armstrong, D. W. Angew Chem. Int. Ed. 2007, 46, 9073-9077.
274. Luo, S., Li, J.; Xu, H.; Zhang, L.; Cheng, J.-P. Org. Lett. 2007. 9, 3675-3678
275. Gao, Q.; Liu, Y., Lu, S.-M., Li, J., Li, C. Green Chem. 2011, 13, 1983-1985.
276. Luo, S.; Li, J.; Zhang, L.; Xu, H.; Cheng, J.-P Chem Eur J 2008, 14, 1273-1281.
277. Demuynck, A. L. W.; Peng, L., de Clippel, F.; et al. Adv. Synth. Catal. 2011, 353, 725-732.
278. Nagendrappa, G. Appl. Clay Sci. 2011, 53, 106-138.
279. Srivastava, V.; Gaubert, K.; Pucheault, M.; Vaultier M. ChemCalChem 2009, 1, 94-98
280. Bergbreiter, D. E.; Tian, J., Hongfa, C. Chem. Rev. 2009, 109, 530-582
281. Plaquevent, J.-C.; Levillain, J.; Guillen, F.; Malhiac, C.; Gaumont, A.-C. Chem. Rev. 2008, 108, 5035-5060.
282. María, P D. d. Angew Chem. Int. Ed. 2008, 47, 6960-6968
283. Winkel, A.; Heddy, P. V. G.; Wilhelm, R. Synthesis 2008, 999-1016.
284. Huo, C.: Chan, T. H. Chem. Soc. Rev. 2010, 39, 2977-3006.
285. Šebesta, R.; Kmentová, I., Toma, Š. Green Chem. 2008. 10, 484-496
286 Nr. B.; Headley A D Chem Eur J 2010, 16, 4426-4436
287. Zhang, W.; Cai C. Chem. Commun. 2008, 5686-5694
288. Durand, J., Teuma, E., Gomez, M. C. R. Chim. 2007, 10, 152-177
289. Paczai, A., Kotschy, A. Monatsh. Chem. 2007, 138, 1115-1123.

    Kotrusz, P.; Kmentová, I.; Gotov, B.; Toma, Š.; Solčániová, E. Chem. Commun. 2002, 2510–2511.
    Lombardo, M.; Easwar S.; Pasi, F.; Trombin, C. Adv. Synth. Catal. 2009, 351, 276–282.
    Gu, Y.; Li, G. Adv. Synth. Catal. 2009, 351, 817–847.

    Gruttadauria, M., Riela, S.; Meo, P. L.; D'Anna, F.; Noto, R. Tetrahedron Lett. 2004, 45, 6113–6116.
    Kucherenko, A. S.; Struchkova, M. L.; Zlotin, S. G. Eur. J. Org. Chem. 2006. 2000–2004.

295. Aprile, C., Giacalone, F.; Gruttadauria, M., et al. Green Chem. 2007, 9, 1328-1334.
```

# EXHIBIT 14

# IN THE UNITED STATES DISTRICT COURT FOR THE DISTRICT OF DELAWARE

NIPPON SHINYAKU CO., LTD.,

Plaintiff,

v.

SAREPTA THERAPEUTICS, INC.,

Defendant.

C.A. No. 21-1015 (GBW)

SAREPTA THERAPEUTICS, INC.,

Defendant/Counter-Plaintiff,

v.

NIPPON SHINYAKU CO., LTD. and NS PHARMA, INC.

Plaintiff/Counter-Defendants.

**DECLARATION OF DR. BRADLEY L. PENTELUTE** 

# **Table of Contents**

				<u>Page</u>			
I.	INTF	RODUC	ΓΙΟΝ	1			
II.	QUA	QUALIFICATIONS					
III.	COM	OMPENSATION AND PRIOR TESTIMONY					
IV.	BAC	KGROU	JND INFORMATION	3			
	A.	NS's '322 Patent					
	B.	Summ	nary of My Task	6			
	C.	Legal Principles					
	D.	Skille	d Artisan	7			
V.	TEC	TECHNICAL INFORMATION					
	A.	PMOs	s Are Chemically Modified Antisense Oligonucleotides	8			
	B.	PMOs Are Made by Connecting Morpholino Monomers One by One in the Synthesis Method Set Forth in the '322 Patent					
		1.	The '322 Patent Uses Morpholino Monomers that Are Protected at Two Different Sites: the Bases and the 3'-Nitrogen Position	11			
		2.	The '322 Patent Uses a Solid-Phase Method	12			
		3.	The '322 Patent Uses a Repeated Cycle of Deprotection and Coupling Reactions to Assemble the PMO	13			
VI.	DISF	UTED (	CLAIM TERMS OF NS'S '322 Patent	16			
	A.	_	e): "reacting said Compound 3 with a deprotecting agent to form bound 4"	16			
		1.	The Claimed Steps, Including Step e), Must Be Performed in the Order Specified in the Claims	17			
		2.	A Skilled Artisan Would Have Understood that Compound 3 Reacts Directly with a Deprotecting Agent	30			
		3.	NS's Construction Is Ambiguous	33			
	B.		): "reacting Compound 4 with an acid to form said oligomer" or ing said Compound 4 with an acid to form said PMO"	34			

# 

1.	The Claimed Steps, Including Step 1), Must Be Performed in the Order Specified in the Claims	35
2.	A Skilled Artisan Would Have Understood that Compound 4 Reacts Directly with an Acid	38
3.	NS's Construction Is Ambiguous	40

I, Bradley L. Pentelute, Ph.D., declare as follows:

# I. INTRODUCTION

1. I have been retained by Sarepta Therapeutics, Inc. ("Sarepta") as an independent expert in the synthesis of antisense oligonucleotides. I understand that Nippon Shinyaku Co., Ltd. and NS Pharma, Inc. ("NS") have asserted claims 1-10 of U.S. Patent No. 10,683,322 ("the '322 patent"; Ex. 2) against Sarepta. I submit this declaration on behalf of Sarepta to offer my opinions regarding the meaning of certain terms that appear in those claims.

# II. QUALIFICATIONS

- 2. I am a tenured professor in the Department of Chemistry at the Massachusetts Institute of Technology ("MIT") in Cambridge, Massachusetts. My research focuses on investigating new chemistry to build and modify macromolecules such as proteins and oligonucleotides.
- 3. I earned my B.S. in Chemistry at the University of Southern California in 2003. In 2004, I received my M.S. in Chemistry from the University of Chicago. In 2008, I earned my Ph.D. in Organic Chemistry also from the University of Chicago. My thesis focused on synthesis of large molecules such as proteins and was titled: New Chemical Methods for the Synthesis of Proteins and the Application to the Elucidation of Protein Structure by Racemic Protein Crystallography. From 2008 to 2011, I was a postdoctoral fellow in the laboratory of Professor R. John Collier in the Department of Microbiology and Molecular Genetics at Harvard Medical School. During this time, I carried out structure-function studies on deciphering how Anthrax toxin enters into host cells.
- 4. I began my independent research career as an Assistant Professor in the Department of Chemistry of MIT in 2011. I was promoted to Associate Professor in 2016 and became a tenured Professor in 2021. In addition to my position within the Department of Chemistry, I also serve as

an Extramural Member in the Koch Institute for Integrative Cancer Research at MIT, an Associate Member of the Broad Institute of MIT and Harvard University, and a Member of the Center for Environmental Health Sciences at MIT.

- 5. I have held several positions external to MIT, including Visiting Professor at the Tokyo Institute of Technology (2019-2020) and Osaka University (2015-2018) in Japan. I have also served as a member of the Nominating Committee for the American Peptide Society since 2015.
- 6. I am the co-founder of Amide Technologies (focuses on the rapid chemical synthesis of proteins), Decoy Therapeutics (focuses on the development of antiviral peptides), and Tegrigen Therapeutics, and the co-scientific founder of Resolute Biosciences (focuses on enhancing the properties of therapeutic proteins by reducing immune reactions) and New Frontier Bio (focuses on natural products for anti-aging). In line with my research interest and expertise, these companies specialize in synthesizing and modifying molecular compounds for therapeutic applications.
- 7. I have authored more than 120 scientific articles, most of which explore synthesis, modifications, functions, and characterizations of biomolecular structures including morpholino oligonucleotides.
- 8. I have also served as an editor and reviewer of several journals in my field of research. Currently, I am an Associate Editor of Nature's *Scientific Reports* and a Guest Editor for the American Chemical Society's *Chemical Reviews*. I review articles for multiple high-impact scientific journals including *Chemical Science*, *ChemBioChem*, and *Journal of the American Chemical Society*. I also served as a grant reviewer for the National Institutes of Health and the National Science Foundation (NSF) in 2018 and 2015, respectively.

- 9. I have been recognized for my research by academia and the pharmaceutical industry. A selection of honors I have received include the American Peptide Society Rao Makineni Lectureship (2021), Eli Lilly Award in Biological Chemistry (2018), Bristol-Myers Squibb Innovation Award (2017), Amgen Young Investigator Award (2016), Novartis Early Career Award in Organic Chemistry (2015), and NSF CAREER Award (2014).
- 10. I am an inventor of more than twenty patents and patent applications, several of which relate to syntheses, chemical modifications, and/or conjugations of proteins and oligonucleotides (including morpholinos).
- 11. My professional qualifications are described in further detail in my curriculum vitae, which is attached as **Appendix A**.

# III. COMPENSATION AND PRIOR TESTIMONY

12. I am being compensated for the time I spend on this matter at a rate of \$400 per hour. My compensation is not contingent upon my opinions offered or the outcome of this matter. I have not testified as an expert in the last four years.

# IV. BACKGROUND INFORMATION

# A. NS's '322 Patent

- 13. NS's '322 patent includes 10 claims. I understand that claims 1 and 6 are independent claims and that claims 2-5 and 7-10 depend from claims 1 and 6, respectively.
- 14. As reproduced below, claim 6 of the '322 patent recites a solid-phase method of making a phosphorodiamidate morpholino oligomer ("PMO"). The method involves six separate steps labeled as step a) through step f):
  - 6. A solid-phase method of making a phosphorodiamidate morpholino oligomer (PMO) that is 100% complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in a

human dystrophin pre-mRNA, wherein the 53rd exon in said human dystrophin pre-mRNA consists of a nucleotide sequence corresponding to SEQ ID NO: 1, wherein said PMO hybridizes to said human dystrophin pre-mRNA with Watson-Crick base pairing, wherein the phosphorodiamidate morpholino monomers of said PMO have the formula:

wherein each of R<sup>2</sup> and R<sup>3</sup> represents a methyl; wherein Base is a nucleobase selected from the group consisting of: uracil, cytosine, thymine, adenine, and guanine; and wherein the 5' end of said PMO has the formula:

said method comprising:

a) providing Compound 1:

wherein T represents trityl, monomethoxytrityl, or dimethoxytrityl; wherein each of  $R^2$  and  $R^3$  represents a methyl; and wherein  $B^P$  is a protected Base;

b) reacting said Compound 1 with an acid to form Compound 2;

- c) reacting said Compound 2 with a morpholino monomer in the presence of a base and a solvent;
- d) repeating steps b) and c) until Compound 3 is complete;

e) reacting said Compound 3 with a deprotecting agent to form Compound 4; and

f) reacting said Compound 4 with an acid to form said PMO:

15. Claim 1 of the '322 patent is similarly directed to a solid-phase method of making an oligomer comprising a PMO. Claim 1 also recites six steps labeled as steps a) through f), except that step f) recites "reacting Compound 4 with an acid to form said oligomer."

# **B.** Summary of My Task

- 16. I have been asked to offer opinions on the meaning of steps e) and f) that appear in the claims of the '322 patent. I have been asked to consider these terms from the perspective of a skilled artisan as of the filing date of the '322 patent. For the purpose of this declaration, I have been asked to assume that the '322 patent was filed on August 31, 2011.
- 17. In forming my opinions, I have considered the language of the claims and the specification. I have considered publications that were available as of August 31, 2011, including the materials cited in this declaration and/or listed in **Appendix B**. I have also considered the proposed interpretations of the claims provided by Sarepta and NS, including a portion of NS's Opening Claim Construction Brief dated January 5, 2023. To the extent I am provided with additional information, including any expert declaration(s) in this case, I reserve the right to modify, supplement, and/or expand my opinions based on that information or any additional information that may be relevant to the case. I also reserve the right to prepare slides or other demonstratives to help illustrate my opinions.

# C. Legal Principles

- 18. Because I am not a legal expert, I do not offer any legal opinions in this declaration. Counsel for Sarepta, however, has explained certain legal principles to me, which I have applied in providing my opinions.
- 19. I have been informed that a claim term of a patent must be interpreted from the perspective of a skilled artisan in the pertinent art at the time of the invention. I understand that the interpretation is primarily based on the "intrinsic evidence," which consists of the patent claims, specification, and prosecution history. I understand that a claim term is given its plain and ordinary meaning, except when the specification provides a specific definition or when the specification or the prosecution history reveal that the inventor applied a different meaning than it would otherwise have to a skilled artisan.
- 20. I understand that extrinsic evidence, e.g., scientific publications, treatises, and dictionaries, may also be considered in interpreting patent claims.

#### D. Skilled Artisan

21. I understand that Sarepta previously challenged the '322 patent in a proceeding at the Patent Office. I understand that in that proceeding, Sarepta explained that skilled artisans in the field typically have the following backgrounds:

a Ph.D. in chemistry, biochemistry, cell biology, genetics, molecular biology, or an equivalent, and several years of experience with antisense oligonucleotides for inducing exon skipping, who: (1) also would have been familiar with methods for making and testing the safety and efficacy of antisense oligonucleotides, both *in vitro* and *in vivo*, and the use of antisense oligonucleotides for inducing exon skipping in the context of medical conditions, such as DMD, that may be treated by administering antisense oligonucleotides and (2) would have had knowledge of and experience with chemical

modifications that may be incorporated into antisense oligonucleotides, such as modifications to the backbone and/or nucleobases of the antisense oligonucleotides, and the potential impact of those modifications on the utility of the antisense oligonucleotides.

22. I agree with this definition and have adopted it in developing my opinions. Unless indicated otherwise, my opinions regarding a skilled artisan apply to such a person as of August 31, 2011, which I understand is the filing date of the '322 patent.

#### V. TECHNICAL INFORMATION

### A. PMOs Are Chemically Modified Antisense Oligonucleotides

- 23. As the name suggests, oligonucleotides are chains of connected subunits called nucleotides. *See* Ex. 5 (Chan 2006) at 533-35. Analogous to genetic materials that exist in nature such as DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), each nucleotide subunit of the oligonucleotide chain has one base attached to it. Ex. 8 (Summerton 1997) at 187, 188 figs.1 & 2. The oligonucleotide chain therefore presents a linear sequence of bases. *Id*.
- 24. Each base can be selected from four types of bases: adenine (A), cytosine (C), thymine (T) (or uracil (U)), and guanine (G). Ex. 4 (Alberts 2002) at 193–95. A specific type of base can "base-pair" with another specific type through a hydrogen-bonding interaction as follows: (i) C with G and (ii) A with T or U. *Id*. The bases of an oligonucleotide can be arranged in order such that they can base-pair with counterpart bases of a target sequence. In that case, the oligonucleotide sequence is considered "complimentary" to the target sequence. The "antisense" in "antisense oligonucleotide" indicates that the base sequence of the oligonucleotide is designed to be complementary to bases found in naturally occurring genetic materials, for example, an RNA molecule. Ex. 8 (Summerton 1997) at 187.

- 25. By August 31, 2011, several research groups were investigating the use of antisense oligonucleotides as therapies. *See*, *e.g.*, Ex. 5 (Chan 2006) at 537-38; Ex. 9 (Summerton 2003) at 233-34. However, it was well known that antisense oligonucleotides synthesized using natural genetic materials are susceptible to degradation in the human body. Ex. 5 (Chan 2006) at 533. Thus, researchers often used chemically modified nucleotides to enhance the stability of antisense oligonucleotides while preserving or enhancing their biological activity. Ex. 5 (Chan 2006) at 535-36; Ex. 8 (Summerton 1997) at 187-88.
- 26. In a typical oligonucleotide, each subunit of the chain, or nucleotide, has three parts—a ring structure, a base attached to the ring structure, and a phosphate group connecting one subunit to the next. *See* Ex. 8 (Summerton 1997) at 187-89. **Figure 1** below illustrates the three parts of an oligonucleotide.

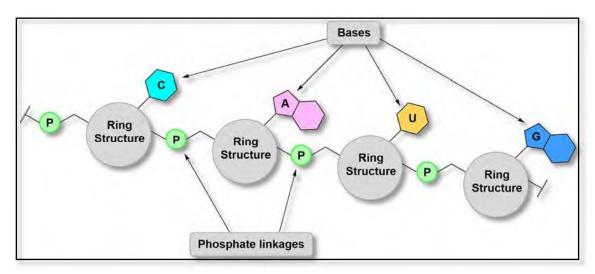


Figure 1. Schematic Drawing of an Oligonucleotide

27. Phosphorodiamidate morpholino oligomers ("PMOs") are one type of antisense oligonucleotide that contain chemically modified nucleotides and were being actively investigated in 2011. Ex. 5 (Chan 2006) at 535-36. **Figure 2** below illustrates chemical differences between PMOs and RNA. For example, RNA has a five-membered ring known as a ribose. In contrast, a PMO has a six-membered ring known as a morpholinyl group (indicated by the blue square). Ex.

5 (Chan 2006) at 546 fig.2. PMO subunits are linked through phosphorodiamidate linkages (indicated by the pink square), instead of phosphate linkages in RNA. *See id*.

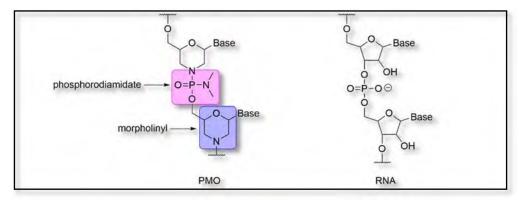


Figure 2. Chemical Structures of PMO and RNA

28. Like naturally occurring nucleotides, PMO monomers have directionality. Ex. 6 (Moulton 2009) at 1305-06. In DNA and RNA, as illustrated in **Figure 3**, the five-membered ring and its exocyclic carbon are numbered clockwise, starting with a carbon next to the oxygen in the ring. In DNA and RNA, individual nucleotides are linked through chemical groups that are bonded to the central ring structure at what are called the 3' and 5' positions. *See id.*; Ex. 4 (Alberts 2002) at 193-95. Although the central morpholine ring in a PMO monomer does not have the same numbering as natural nucleotides, researchers have adopted the 3' and 5' nomenclature by analogy. Ex. 6 (Moulton 2009) at 1305-06. **Figure 3** shows the numbering and directionality of RNA and a PMO.

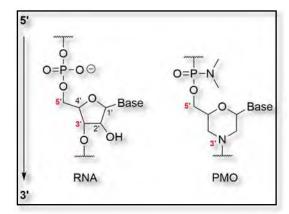


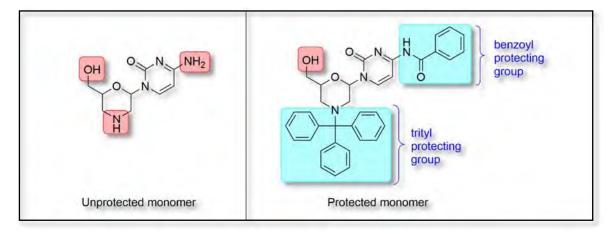
Figure 3. Numbering and Directionality of RNA and a PMO

# B. PMOs Are Made by Connecting Morpholino Monomers One by One in the Synthesis Method Set Forth in the '322 Patent

29. The biological activity and therapeutic benefits of antisense oligonucleotides including PMOs derive from their sequential ordering of bases. Ex. 8 (Summerton 1997) at 187. As such, an antisense oligonucleotide is typically assembled through a series of sequential couplings of subunits that collectively make up its base sequence. Ex. 7 (Pon 2000) at 3.1.1. As discussed below, NS's method claimed in the '322 patent similarly involved cycling through a sequence of chemical reactions to build a chain of PMO subunits with the desired length and base sequence.

# 1. The '322 Patent Uses Morpholino Monomers that Are Protected at Two Different Sites: the Bases and the 3'-Nitrogen Position

30. During each cycle of a PMO-lengthening reaction, each newly added morpholino monomer is attached to the previous subunit through a phosphorodiamidate group. However, morpholino monomers have more than one chemically reactive site, which potentially causes undesired chemical reactions. As shown in **Figure 4** below, an unprotected morpholino monomer may have several chemically reactive sites (shown in red boxes).



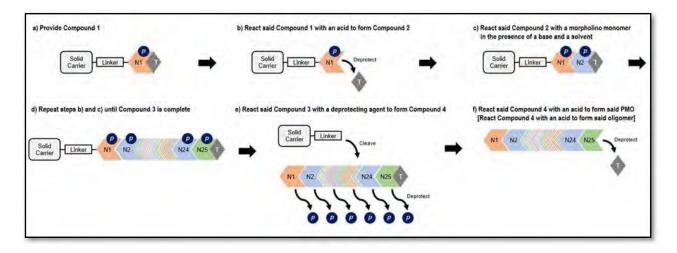
**Figure 4**. Chemical Protection Strategy for Morpholino Monomers (Adapted from Ex. 2 at 19:4-20)

31. To ensure that the desired bonds form between the correct chemical groups (i.e., the 5'-exocyclic carbon of one subunit and the 3'-morpholino nitrogen of another), the other reactive sites on the morpholino monomer are protected, i.e., one or more sites of the monomer are attached to a protecting group that prevents the sites from participating in chemical reactions. In the case of the synthesis scheme set forth in the NS patent and as shown in **Figure 4** above, each monomer is protected at two sites. Ex. 2 at 21:6-25. The 3'-morpholino nitrogen on the monomer is protected with a protecting group selected from a trityl group (shown in the lower blue box), a monomethyoxytrityl group, or a dimethoxytrityl group. *Id.* at 15:23-24. In the claims, these protecting groups are represented by "T." *Id.* at claims 1 & 6. Reactive groups on the nucleobases are also chemically protected using, for example, a benzoyl protecting group (shown in the upper blue box). *Id.* at 15:34-52. In the claims, each of the protected bases is represented by "B<sup>P</sup>." *Id.* at claims 1 & 6.

### 2. The '322 Patent Uses a Solid-Phase Method

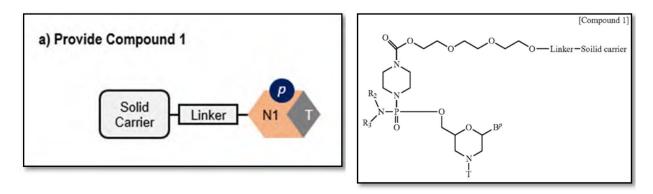
32. The '322 patent uses a solid-phase method, meaning that a solid support such as a resin is used to support PMO synthesis. Ex. 2 at 15:53-16:15. The growing chain of the PMO is covalently linked to the solid support, referred to as a "solid carrier" in the claims. *Id.* at claims 1 & 6. The covalent linkage is referred to as a "linker" in the claims, and the specification indicates that "[a] 'linker' which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives," including "3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA)." *Id.*; *see also id.* at 16:16-20.

- 3. The '322 Patent Uses a Repeated Cycle of Deprotection and Coupling Reactions to Assemble the PMO
- 33. As noted above, the synthesis methods set forth in the '322 patent generate a PMO by attaching one morpholino subunit to another one in a sequential manner. **Figure 5** below is a schematic drawing of this method.



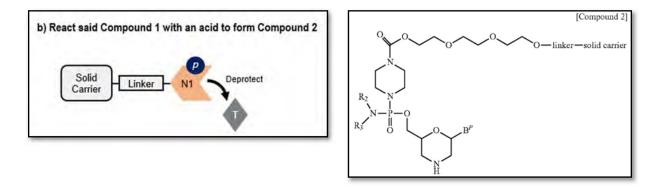
**Figure 5**. Solid-Phase Synthesis Scheme for PMOs Set Forth in the '322 Patent (*See* Ex. 2 at claims 1 & 6; graphic adapted from Ex. 7 (Pon 2000) at 3.1.2 fig.3.1.1.)

34. <u>Step "a) providing Compound 1"</u>: The '322 patent starts with a first morpholino monomer ("N1" in **Figure 5**), which is chemically attached to a solid carrier via a chemical linker. This linked monomer is referred to as Compound 1 in NS's claims.

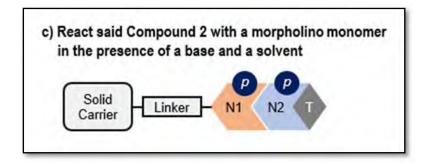


35. Step "b) reacting said Compound 1 with an acid to form Compound 2": In order to connect the first monomer to the second monomer in the chain, the protecting group attached to

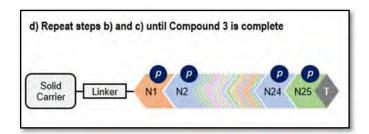
the 3'-morpholine nitrogen is removed, i.e., deprotected. The '322 patent discloses using an acid to remove this protecting group, and the resulting deprotected monomer is referred to as Compound 2.

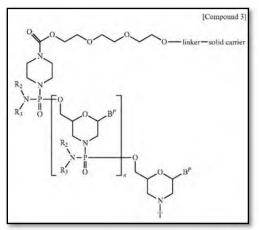


36. Step "c) reacting said Compound 2 with a morpholino monomer in the presence of a base and a solvent": The second monomer ("N2" in **Figure 5**) is then added, and the exposed nitrogen of the first monomer reacts with and links to the second monomer, i.e., is coupled. The '322 patent carries out this reaction in the presence of a base and a solvent.

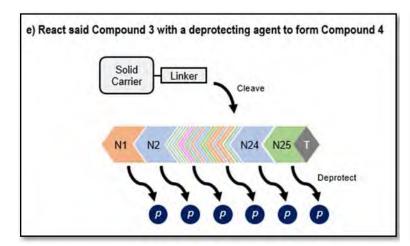


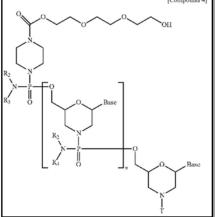
37. Step "d) repeating steps b) and c) until Compound 3 is complete": The deprotection and coupling steps, steps b) and c), respectively, are then repeated to lengthen the PMO chain one monomer at a time. In the '322 patent claims, this cycling of deprotection and coupling steps continues until the desired number of monomers are included, for example, 25 monomers. This chain of PMO monomers is referred to as Compound 3.



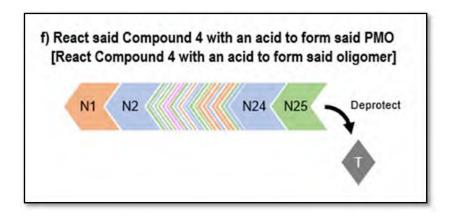


38. Step "e) reacting said Compound 3 with a deprotecting agent to form Compound 4": The '322 patent then uses a deprotecting agent to both (1) cleave the assembled chain of PMO monomers from the solid carrier and (2) deprotect the bases of the PMO monomers in the chain. This cleaved, base-deprotected chain of PMO monomers is referred to as Compound 4.





39. <u>Step "f) reacting [said] Compound 4 with an acid to form said oligomer [or PMO]":</u> The 3'-morpholine nitrogen in Compound 4 is still protected with a protecting group such as a trityl group. As the final step, the '322 patent removes this protecting group from the assembled chain of PMO monomers using an acid, resulting in the claimed PMO.



#### VI. DISPUTED CLAIM TERMS OF NS'S '322 PATENT

A. Step e): "reacting said Compound 3 with a deprotecting agent to form Compound 4"

Term	NS's Position	Sarepta's Position
"e) reacting said	Plain and ordinary meaning—i.e.,	Plain and ordinary meaning, i.e.,
Compound 3 with a	chemically reacting Compound 3	chemically reacting a deprotecting
deprotecting agent	with a deprotecting agent, in order	agent directly with Compound 3 of
to form Compound	to form Compound 4	step d), which results in
4"	_	Compound 4

- 40. Step e) of each of claims 1 and 6 requires "reacting said Compound 3 with a deprotecting agent to form Compound 4." *See supra* ¶13-15. Sarepta proposes interpreting this step to have its plain and ordinary meaning, i.e., "chemically reacting a deprotecting agent directly with Compound 3 of step d), which results in Compound 4." This interpretation incorporates the order of steps performed, i.e., using Compound 3 produced in the prior step d) to form Compound 4 (which is then used in step f)). It also specifies the nature of this reaction, i.e., Compound 3 reacts directly with a deprotecting agent. NS's interpretation, in contrast, does not indicate the order of the steps or the nature of the reaction in step e).
- 41. As discussed below, a skilled artisan would have understood that the steps of the claims, including step e), must be performed in the order specified in the claims. A skilled artisan would have also understood that the reaction in step e) reacts Compound 3 directly with a deprotecting agent. The skilled artisan's understanding is consistent with Sarepta's interpretation.

In contrast, NS's interpretation departs from that understanding and suffers from multiple deficiencies. As such, it is my opinion that Sarepta's construction should be adopted.

- 1. The Claimed Steps, Including Step e), Must Be Performed in the Order Specified in the Claims
  - a. The Claim Language
- 42. A skilled artisan reading the claims would have concluded that the steps must be performed in the order listed in the claims. A plain reading of the claims supports that conclusion. Below, I use claim 6 of the '322 patent to illustrate the express sequential linkage among the steps:
  - a) providing Compound 1 ...;
    b) reacting said Compound 1 with an acid to form Compound 2
    c) reacting said Compound 2 with a morpholino monomer in the presence of a base and a solvent;
    d) repeating steps b) and c) until Compound 3 is complete;
    e) reacting said Compound 3 with a deprotecting agent to form

6.1 A solid-phase method of making a phosphorodiamidate morpholino

- f) reacting said Compound 4 with an acid to form said PMO: . . . .
- 43. Claim 6 starts with step a), in which Compound 1, a solid carrier linked morpholino monomer, is provided. Following step a), each step refers back to the particular compound made in the prior step or (in case of step d)) expressly refers back to the prior steps. Using that compound, each step leads to the formation of a new compound used in the next step or (in case of step f)) the claimed PMO. For example, step b) refers back to Compound 1 provided in step a)

<sup>&</sup>lt;sup>1</sup> The chemical structures of Compounds 1-4 have been omitted.

by expressly identifying it with "said" Compound 1.<sup>2</sup> Step b) results in Compound 2, which is then used in step c). Step c) again refers back to Compound 2 made in step b) by expressly identifying it with "said" Compound 2. In view of the express claim language linking each step sequentially, a skilled artisan would have understood that the steps must be performed in the order specified in the claims.

- 44. Moreover, as illustrated above, the claims use both alphabetical order in listing steps a) through f) and numerical order in reciting Compounds 1 through 4. A skilled artisan would consequently interpret these steps as describing a particular sequence of steps that should be carried out in the order written. Nothing in the '322 patent suggests that a skilled artisan should ignore this sequence of steps.
- 45. Step e) is not an exception. Step e) refers back to Compound 3 formed in step d) by expressly identifying it with "said" Compound 3. The reaction in step e) uses that compound to "form Compound 4," which in turn is used in step f). Based on the plain language of step e), it is logical to conclude that step d) is performed before step e) to obtain *said* Compound 3 used in step e). It is also logical to conclude that the reaction in step e) results in Compound 4; otherwise, step f) that uses *said* Compound 4 cannot be performed. Sarepta's reading of step e) captures this logical order of steps, specifying that Compound 3 used in step e) is "of step d)" and the reaction of step e) "results in Compound 4."

previously").

<sup>&</sup>lt;sup>2</sup> I have been informed that "said" in patent law refers to what has been mentioned before, akin to "the" used in the same context. This is consistent with my understanding of the plain meaning of "said." *See*, *e.g.*, Ex. 10 at 1443 (*Collins English Dictionary* defining "said" as "mentioned

# b. The Specification

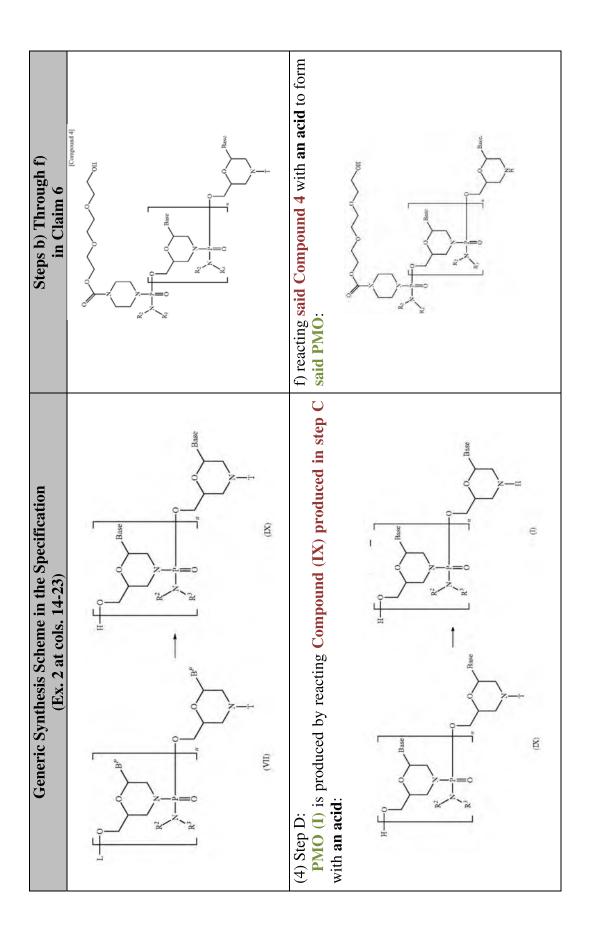
46. The specification also supports my opinion that a skilled artisan would have concluded that the steps, including step e), are performed in the order identified in the claims. Two portions of the specification are relevant to my analysis. First, in the section titled "Method for Producing PMO," the specification discloses a generic synthesis scheme for making a PMO. Ex. 2 at cols. 14-23. This is the *only* generic synthesis scheme described in the specification.<sup>3</sup> Second, in Example 1, the specification discloses a synthesis scheme used to make a PMO referred to as PMO No. 8. *Id.* at cols. 31-33. Again, this is the *only* example that provides specific synthesis reactions and conditions used to make a PMO tested in the specification's other examples.

D, which uses somewhat different nomenclature than the corresponding steps of the claims (steps b) through f)). Ex. 2 at cols. 14-23. Nevertheless, as illustrated below, Step A in the specification corresponds to step b) of the claims, Step B in the specification corresponds to step c) of the claims, the repetition of Step A and Step B in the specification corresponds to step d) of the claims, Step C in the specification corresponds to step e) of the claims, and Step D in the specification corresponds to step f) of the claims. Comparing the steps in the claims with the specification's generic synthesis scheme confirms that the specification's generic synthesis scheme follows the precise order of steps specified in the claims with no modified or inserted steps that transform the claimed compounds to different compounds.

<sup>&</sup>lt;sup>3</sup> The specification indicates that this scheme can be carried out "by the liquid phase method or the solid-phase method." Ex. 2 at 14:38-43. Because the claims of the '322 patent are limited to a "solid-phase method," I have focused on the disclosures related to a solid-phase method.

Generic Synthesis Scheme in the Specification (Ex. 2 at cols. 14-23)	Steps b) Through f) in Claim 6
	b) reacting said Compound 1 with an acid to form Compound 2;  [Compound 2]  [Compound 2]
solid carrier linker	
(2) Step B:  Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):	c) reacting said Compound 2 with a morpholino monomer in the presence of a base and a solvent;

Steps b) Through f) in Claim 6		d) <b>repeating steps b) and c)</b> until Compound 3 is complete;	R <sub>2</sub> N N N N N N N N N N N N N N N N N N N	Z-i-	e) reacting said Compound 3 with a deprotecting agent to form Compound 4; and
Generic Synthesis Scheme in the Specification (Ex. 2 at cols. 14-23)	I.—O BP monthlolino monthlolin	In Compound (II), the compound represented by general formula (IIa2) can be produced by repeating step A and step B of the PMO production method described in the specification for a desired number of times	(IIa2) solid currier linker O BP R3 N P O BP	Z	(3) Step C: In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



- 49. The specification's discussion of Step C, which corresponds to step e) of the claims, is particularly notable. In discussing Step C, the specification instructs a skilled artisan to use "Compound (VII) *produced in* Step B." Ex. 2 at 22:7-67. As noted above, "Step B" and its repetition in combination with Step A correspond to step d) of the claims, while "Compound (VII)" corresponds to Compound 3 of the claims. Stated differently, in discussing step e) of the claims, the specification instructs a skilled artisan to use Compound 3 "produced in" step d). This express instruction is consistent with Sarepta's interpretation that identifies step d) as the source of Compound 3 for use in step e).
- 50. The sole example in the specification, Example 1, similarly follows the identical order of steps written in the claims with no modified or inserted steps that transform the claimed compounds to different compounds.

Example 1 in the Specification (Ex. 2 at cols. 31-33)	Steps a) Through f) in Claim 6
4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmop was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. <sup>4</sup>	a) providing Compound 1:  [Compound 1]  O O O O Linker-Soilid carrier  N N P N P T

<sup>&</sup>lt;sup>4</sup> PMO No. 8 synthesized in Example 1 has -OH at its 5'-end whereas the PMOs made in accordance with claims 1 and 6 of the '322 patent have a triethylene glycol at their 5'-end. Example 11 discusses synthesizing PMO No. 13, which has a triethylene glycol at its 5'-end and indicates that it was "produced in accordance with the procedure of EXAMPLE 1." Ex. 2 at 34:58-67. In other words, the specific reaction conditions and order of reactions in Example 1 did not differ for making a PMO having the same 5'-end group set forth in the claims.

# Example 1 in the Specification (Ex. 2 at cols. 31-33)

The desired morpholino monomer compound was added in each **cycle** to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (mm)
1	deblocking solution	30	2.0
2 3	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
_15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6-11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromethane	30	0.5
25	dichloromethane	30	0.5

The deblocking solution used was a solution obtained by dissolving a mixture trifluoroacetic acid (2 equivalents) and triethylamine equivalent) (1 dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N, Ndiisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained dissolving by the morpholino monomer compound in 1,3dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15 M. The coupling solution B used was a solution obtained by dissolving **diisopropylethylamine** in 1,3-dimethyl-2imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2,6-lutidine in dichloromethane.

# Steps a) Through f) in Claim 6

b) reacting said Compound 1 with an acid to form Compound 2;

[Compound 2]

$$O \longrightarrow O \longrightarrow O \longrightarrow O$$
 $O \longrightarrow O \longrightarrow O$ 
 $O \longrightarrow O \longrightarrow O$ 

c) reacting **said Compound 2** with a morpholino monomer in the presence of **a base** and a solvent;

d) repeating steps b) and c) until Compound 3 is complete;

### **Example 1 in the Specification** Steps a) Through f) (Ex. 2 at cols. 31-33) in Claim 6 The aminomethyl polystyrene resin loaded e) reacting said Compound 3 with a with the PMO synthesized above was deprotecting agent to form Compound 4; and recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55° C. for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. . . Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL of f) reacting said Compound 4 with an acid to 10 mM phosphoric acid aqueous solution to form said PMO: suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45 µm). The

51. In sum, in describing the solid-state synthesis method, the specification follows the precise order of steps as written in the claims. Nothing in the specification tells a skilled artisan to deviate from that order. Reading the claims in light of the specification, a skilled artisan would have concluded that the steps in the claims, including step e), must be performed in the order written.

mixture was thoroughly washed . . . to give the

product as an aqueous solution.

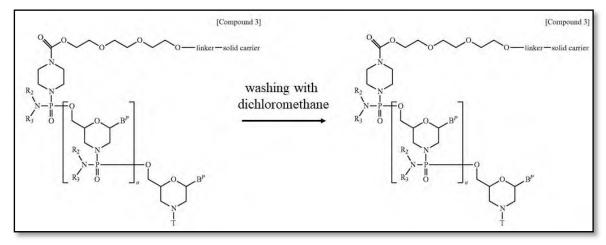
## c. NS's Disagreement with Sarepta's Construction

# i. "Comprising"

- 52. Each of claims 1 and 6 recite that the claimed method "compris[es]" steps a) through f). I understand that the term "comprising" in patent law is an open transitional phrase, meaning that the recited elements are essential but other elements may be added. In the case of the claims of the '322 patent, NS appears to believe that: (1) Sarepta's construction excludes all possible steps that are not identified in the claims and (2) it is therefore inconsistent with the claims reciting a solid-phase method "comprising" steps a) through f). I disagree.
- 53. A skilled artisan reading the claims would conclude that steps a) through f) must be performed in the order specified because those steps are linked via particular chemical compounds produced in a prior step and used in a next step. *See supra* ¶42-45. The order of the steps is also clear from the specification's sole example. *See supra* ¶46-51. That said, a skilled artisan also would have concluded that the synthesis scheme permits additional steps other than those of steps a) through f), provided that those additional steps do not transform the chemical structure of each recited compound. This is consistent with the additional, exemplary steps discussed in the specification.
- 54. The general synthesis scheme and Example 1 in the specification both include additional, optional steps beyond steps a) through f), such as washing, neutralization, acylation, and purification steps. *See*, *e.g.*, Ex. 2 at cols. 14-24 ("Method for Producing PMO") and 31-33 (Example 1). Notably, these optional steps as used in the specification do *not* transform the chemical structure of the Compounds recited in steps a) through f). For example, the specification uses washing steps between deprotection and coupling. *See id.* at cols. 31-32 (Table 3) steps 13-15; *see also id.* at 31:35-43 ("wash[ing]" the first morpholino monomer attached to a solid carrier using dichloromethane prior to the chain growth reactions). While these washing steps remove

unreacted impurities from the reaction mixture, they do not affect the chemical structure of the recited Compounds.

- 55. Similarly, the specification also discusses an acylation step, which a chemist can optionally use after coupling a morpholino monomer to the growing chain of PMO monomers. *See id.* at 21:46-47 ("Furthermore, after completion of this step, an acylating agent can be added, if necessary."); *id.* at cols. 31-32 (Table 3) steps 21 and 22 ("capping"). This step allows an acylating agent to react with any unreacted PMO (i.e., any chain that did not react with the added morpholino monomer) such that the unreacted PMO no longer participates in the next round of coupling. This is sometimes referred to as "capping" of the unreacted starting material. Again, this acylation step does not alter the chemical structure of a compound properly coupled with the added morpholino monomer.
- 56. Consistent with these additional steps in the specification, Sarepta's construction of step e) allows non-transformative steps, i.e., steps that do not transform the chemical structure of Compound 3 to another compound. For example, as illustrated below, a washing step can be implemented after step d) and before step e) because washing does not alter the chemical structure of Compound 3 made from step d) and used in step e).



**Figure 6**. Washing Step Used with Compound 3 (Schematic Representation of Washing Steps Used in Example 1)

57. Adding an acylating agent also does not alter the chemical structure of Compound 3. An acylating agent such as acetic anhydride is added to append an acyl group ("Ac" as highlighted in blue below) to any unreacted starting material, i.e., a chain of PMOs with an unprotected 3'-nitrogen that did not couple with the next intended morpholino monomer ("n-1" as highlighted in yellow). Because the acylated compound does not participate in the remaining steps of the claimed method, this acylating step ensures the sequence integrity of the final product. Acylation does not chemically transform Compound 3. Instead, it modifies incomplete chains of PMO monomers that can be subsequently separated from the desired PMO.

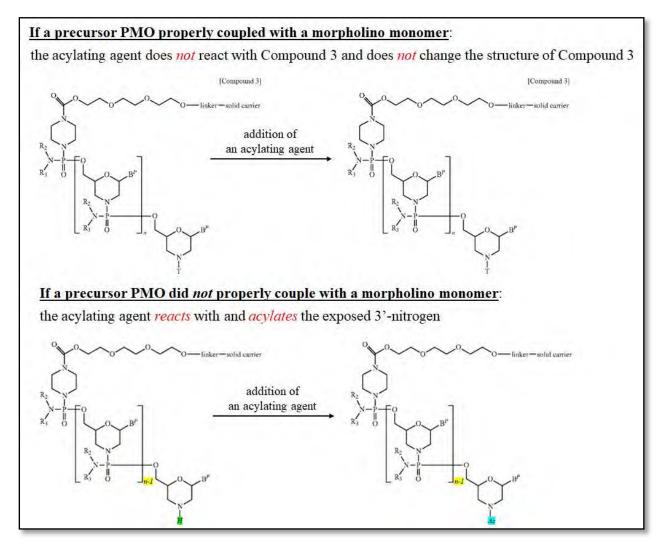


Figure 7. Acylating Step Used with Compound 3

58. In sum, the plain claim language requires the steps to be performed in the order specified because of the language and logic of the claims. When read in light of the specification, a skilled artisan would have understood that the claims allow additional, optional steps that do not transform the chemical structure of the compounds of steps a) through f). Consistent with a skilled artisan's understanding, Sarepta's interpretation of the claims does not exclude these steps, and therefore is consistent with the claimed methods "comprising" steps a) through f).

#### ii. NS's Hypothetical Scenario

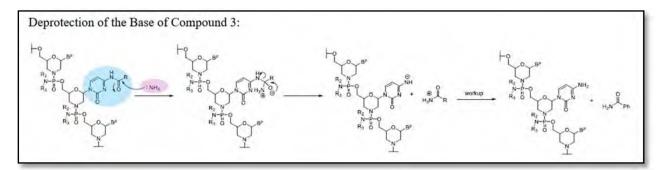
59. NS relies on a hypothetical scenario to argue that Sarepta's construction is incorrect. As illustrated below, unlike the steps of the claims, the steps used in NS's hypothetical do not reference "said" compound. Thus, NS's hypothetical ignores important claim limitations, and it does not help me understand NS's claims.

Step Number	Method A (Only Claimed Steps)	Method B (Additional Unrecited Steps)
1 R	Step a) – Providing Compound 1  cacting said Compound 1 and	Step a) – Providing Compound 1  Reacting said Compound 1 and
2	Step b) – Forming Compound 2	Step b) - Forming Compound 2
3	Step c) – Reacting Compound 2 with a monomer	Step c) – Reacting Compound 2 with a monomer
4	Step d) – repeating steps b) and c) to form Compound 3	Step d) – repeating steps b) and c) to form Compound 3
5		Reacting Compound 3 with a reagent to form an intermediate.
6	said	Reacting the intermediate with a second reagent to re-form Compound 3.
7	Step e) – Reacting Compound 3 with reagent to form Compound 4	Step e) – Reacting Compound 3 with reagent to form Compound 4
8	Step f) – Reacting Compound 4 with acid to form the Oligomer or PMO	Step f) – Reacting Compound 4 with acid to form the Oligomer or PMO

Figure 8. Missing Claim Limitations in NS's Hypothetical Scenario

# 2. A Skilled Artisan Would Have Understood that Compound 3 Reacts Directly with a Deprotecting Agent

- 60. Step e) of claims 1 and 6 requires "reacting said Compound 3 with a deprotecting agent to form Compound 4." A skilled artisan familiar with this reaction would have understood that Compound 3 reacts *directly* with a deprotecting agent.
- 61. A chemical reaction typically involves different chemical compounds. Some compounds interact with each other to transform the chemical structures of those compounds. Other compounds do not participate in the chemical transformation but facilitate the process, for example, solvents and diluents that create an environment where the transformation can take place.
- 62. Compound 3 and the "deprotecting agent" in step e) fall into the former category of compounds that chemically react with each other. The following chemical reaction schemes, discussed below, illustrate how these two compounds react directly with each other to create a new compound.



**Figure 9**. Deprotection of the Base of Compound 3

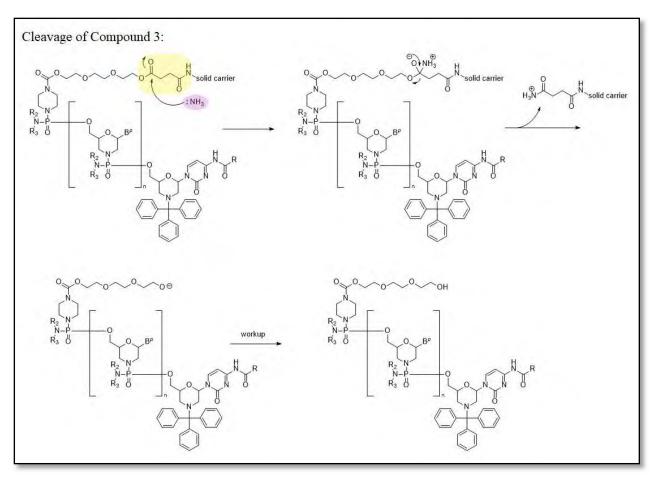


Figure 10. Cleavage of Compound 3 From a Solid Carrier

63. In the first reaction (**Figure 9**), I have used ammonia ("NH<sub>3</sub>"; highlighted in purple) disclosed in the '322 patent as an exemplary deprotecting agent. *See* Ex. 2 at 22:49-50. Here, ammonia reacts directly with the chemically protected cytosine (highlighted in blue) of a PMO attached to a solid carrier (corresponding to Compound 3 in step e)). This direct reaction removes the protecting group from cytosine. In the second reaction (**Figure 10**), I have used a succinyl linker (highlighted in yellow) disclosed in the '322 patent as an exemplary linker. *See id.* at 16:16-20. Again, ammonia reacts directly with the succinyl linker, cleaving the PMO from the solid carrier. Collectively, these direct chemical reactions transform Compound 3 to Compound 4 (**Figure 11**). Sarepta's interpretation of the claims reflects the nature of these chemical reactions by specifying that Compound 3 reacts directly with the deprotecting agent.

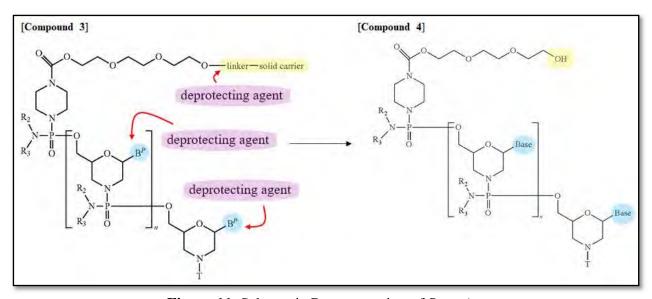


Figure 11. Schematic Representation of Step e)

64. NS argues that Sarepta's "directly" construction is improper. According to NS, a skilled artisan reading Sarepta's "directly" construction would interpret that step e) excludes the presence of other reagents or ingredients used as part of the reaction. I disagree. As explained above, a skilled artisan would have understood that Compound 3 and a deprotecting agent in step e) react directly with one another, causing the chemical transformation of Compound 3 to Compound 4, and that Sarepta's "directly" construction captures this relationship between Compound 3 and the deprotecting agent. This is consistent with how "directly" is used in the scientific literature in describing similar chemical reactions. See, e.g., Ex. 13 at 274-75 (describing a "direct" reaction where a first compound "directly reacts with" a second compound and distinguishing it from an "indirect" reaction where the first compound is converted to another compound, which then reacts with the second compound). A skilled artisan would not have understood that Sarepta's "directly" construction precludes the use of additional chemicals. Id. (noting that the "direct" reaction occurs "in the presence of a catalyst"). Indeed, I am not aware of any chemistry articles or textbooks that use "directly" in the manner proposed by NS, and I understand that NS has cited none.

# 3. NS's Construction Is Ambiguous

- 65. NS proposes interpreting step e) to mean "chemically reacting Compound 3 with a deprotecting agent, in order to form Compound 4." In my view, NS's construction is flawed.
- 66. NS's interpretation does not specify that Compound 3 used in step e) is from step d). Under NS's construction, it is unclear whether step e) allows Compound 3 to be provided from other sources. NS's construction may also allow unrecited steps to be inserted between steps d) and e), even if those steps are chemically transformative, i.e., altering the chemical structure of Compound 3 formed in step d). In my opinion, expanding step e) to encompass Compound 3 from other sources or other steps that chemically transform Compound 3 is contrary to the claim language and the specification. As discussed above, the claims expressly instruct a skilled artisan to use "said Compound 3," referring back to Compound 3 formed in step d). See supra ¶¶42-45. The specification similarly explains that step e) reacts a deprotecting agent with Compound 3 "produced in" step d). See supra ¶¶46-51; Ex. 2 at 22:7-67.
- 67. NS's construction also indicates that the reaction of step e) is performed "in order to form Compound 4." Under NS's construction, Compound 4 appears to be a goal of the reaction rather than a result that must be achieved. The claims, however, state that the reaction of step e) must "form Compound 4." See supra ¶13-15, 42-45. Requiring the formation of Compound 4 is also logical. The claims state that step f) uses said Compound 4 produced in step e). The specification also instructs a skilled artisan to use Compound 4 "produced in" step e). See supra ¶46-51; Ex. 2 at 23:1-56. Without obtaining Compound 4 in step e), step f) cannot be performed.
- 68. In addition, NS's construction does not specify that Compound 3 and a deprotecting agent react directly. This omission, in my opinion, makes NS's interpretation ambiguous. Without it, it is unclear whether the chemical transformation of Compound 3 is caused by the deprotecting agent or some other compound(s). This would potentially expand step e) to reactions not

mentioned in the claims or the specification. For example, under NS's interpretation, step e) could cover a multi-step reaction in which Compound 3 reacts with an unrecited compound X, forming another unrecited compound Y, which then reacts with a deprotecting agent to form Compound 4. This is not how a skilled artisan would have understood the reaction of step e). *See supra* ¶¶60-64. Nor is it how the specification describes step e). The specification only describes a direct reaction between Compound 3 and a deprotecting agent. *See supra* ¶¶46-51; Ex. 2 at 22:7-67.

B. Step f): "reacting Compound 4 with an acid to form said oligomer" or "reacting said Compound 4 with an acid to form said PMO"

Term	NS's Position	Sarepta's Position
"f) reacting	Plain and ordinary meaning—i.e.,	Plain and ordinary meaning, i.e.,
Compound 4 with	chemically reacting Compound 4	chemically reacting an acid
an acid to form said	with an acid, in order to form the	directly with Compound 4 of step
oligomer"	oligomer [or the PMO]	e), which results in the oligomer or
		the PMO.
"f) reacting said		
Compound 4 with		Step f) must occur after step e).
an acid to form said		
PMO"		

69. Step f) of claim 6 states "reacting said Compound 4 with an acid to form said PMO." *See supra* ¶¶13-14. Step f) of claim 1 states "reacting Compound 4 with an acid to form said oligomer." *See supra* ¶15. The differences between Sarepta's and NS's constructions with respect to step f) are similar to those for step e). Specifically, Sarepta proposes that step f) has its plain and ordinary meaning, i.e., "chemically reacting an acid directly with Compound 4 of step e), which results in the oligomer or the PMO." This interpretation incorporates the order of steps performed, i.e., using Compound 4 produced in step e) to form the PMO or oligomer. It also specifies the nature of this reaction, i.e., Compound 4 reacts directly with an acid to form the PMO. NS's interpretation does not specify the order of the steps or the nature of the reaction in step f).

70. As previously discussed, a skilled artisan would have understood that the steps of the claims, including step f), must be performed in the order specified. A skilled artisan would have also understood that the reaction in step f) reacts Compound 4 directly with an acid. Sarepta's interpretation is consistent with how a skilled artisan would read the claims. NS's interpretation departs from that understanding and suffers from multiple deficiencies. As such, my opinion is that Sarepta's construction should be adopted.

# 1. The Claimed Steps, Including Step f), Must Be Performed in the Order Specified in the Claims

### a. The Claims and Specification

- 71. A skilled artisan would have concluded that step f) of claims 1 and 6 must be performed in the order specified. For claim 6, step f) states "reacting said Compound 4 to form said PMO." See supra ¶42-44. Based on the plain language of step f), it is logical that step e) is performed before step f) to obtain "said Compound 4" used in step f). It is also logical that step f) occurs after step e), as step f) results in "said PMO"—the product that the claimed method generates. The specification likewise instructs a skilled artisan to use Compound 4 "produced in" step e). See supra ¶46-51; Ex. 2 at 23:1-56. Sarepta's interpretation of step f) captures the logical order of the steps, specifying that Compound 4 used in step f) is "of step e)" and the reaction of step f) "results in the PMO or oligomer."
- 72. It does not matter that step f) of claim 1 does not refer to "said" Compound 4, for several reasons. First, the overall structure of claim 1 supports that the steps of the claim, including step f), must be performed in the specified order. The steps in the claim repeatedly refer back to the particular compound made in the prior step by referencing "said" compound. Those compounds are then used in a chemical reaction to form a new compound, which in turn is used in the next step. This structure indicates to a skilled artisan that the claimed method as a whole,

including step f), should be performed in the order specified in the claim. *See supra* ¶¶42-44. Second, step f) of claim 1 states that the reaction must "form the oligomer." See supra ¶¶13-15. Logically, it makes sense to place step f) after step e), as it is the final step of the claimed method and yields "the oligomer." Third, this is consistent with the specification's instruction to use Compound 4 "produced in" step e), again placing step f) after step e). See supra ¶¶46-51; Ex. 2 at 23:1-56. Sarepta's interpretation clarifies the order by specifying that "step f) must occur after step e)."

#### b. NS's Disagreement with Sarepta's Construction

# i. "Comprising"

- 73. As with Sarepta's construction of step e), NS disagrees with Sarepta's construction of step f), arguing that it is inconsistent with the claims reciting a method "comprising" steps a) through f). I disagree. *See supra* ¶¶52-58.
- A skilled artisan would have understood that while the claims require a particular sequence of steps, they do not exclude additional, optional steps that do not transform the chemical structure of the compounds specified in steps a) through f). See supra ¶52-58. The specification supports this understanding. For example, the specification discusses additional, optional steps such as washing, neutralization, acylation, and purification. See supra ¶54-57. As used in the specification, none of these additional steps transforms the chemical structures of the compounds recited in the claimed method. See supra ¶54-57. Applying a washing step after step f), for example, does not change the structure of the PMO generated. See, e.g., Ex. 2 at 32:65-67 (Example 1 explaining that the assembled PMO "was thoroughly washed with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution"). Likewise, purifying the PMO after step f) does not change the structure of the PMO generated. See, e.g., id.

- at 33:1-2 ("The resulting aqueous solution containing the product was purified by an anionic exchange resin column.").
- 75. Sarepta's interpretation of step f) is consistent with a skilled artisan's understanding. Sarepta's interpretation of step f) permits additional unrecited steps that do not transform the chemical structure of Compound 4 or the PMO. Thus, Sarepta's interpretation is entirely consistent with the claimed methods "comprising" steps a) through f).

#### ii. NS's Hypothetical Scenario

76. Similar to step e), NS again presented a hypothetical scenario, arguing that Sarepta's interpretation of the claims improperly excludes Method B from the claims. NS's hypothetical again fails. Again, this hypothetical fails because it omits important claim limitations, including "said" compound in several steps.

Step Number	Method A (Only Claimed Steps)	Method B (Additional Unrecited Steps)
1 Reactin	Step a) – Providing Compound 1 g said Compound 1 and	Step a) – Providing Compound 1 Reacting said Compound 1 and
2	Step b) Forming Compound 2	Step b) Forming Compound 2
3	Step c) – Reacting Compound 2 with a monomer	Step c) – Reacting Compound 2 with a monomer
4	Step d) – repeating steps b) and c) to form Compound 3	Step d) – repeating steps b) and c) to form Compound 3
5	Step e) – Reacting Compound 3 with reagent to form Compound 4	Conducting a series of reactions (not a single reaction using a deprotecting agent) to form Compound 4.
6	Step f) – Reacting Compound 4 with acid to form the Oligomer or PMO	Step f) – Reacting Compound 4 with acid to form the Oligomer or PMO
7		Conducting a series of reactions to re- form Compound 3
8		Step e) – Reacting Compound 3 with reagent to form Compound 4
9		Conducting a series of reactions (not a single reaction using an acid) to form the Oligomer or PMO.

Figure 12. Missing Claim Limitations in NS's Hypothetical Scenario

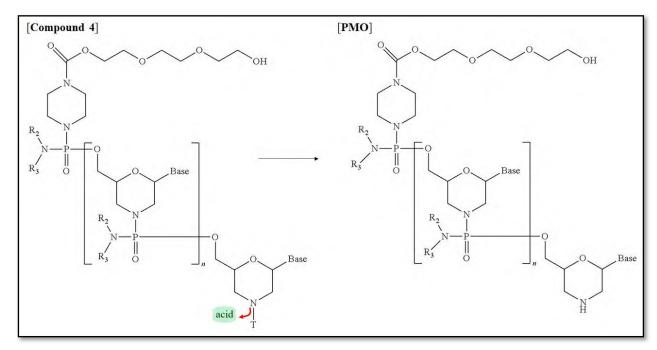
# 2. A Skilled Artisan Would Have Understood that Compound 4 Reacts Directly with an Acid

- 77. Step f) of claim 1 requires "reacting Compound 4 with an acid to form said oligomer." Similarly, Step f) of claim 6 requires "reacting said Compound 4 with an acid to form said PMO." In both cases, a skilled artisan would have understood that Compound 4 reacts *directly* with an acid to form the product.
- 78. As discussed above, different types of chemical compounds may be involved in a chemical reaction. *See supra* ¶61. Some compounds react directly with one another to cause a chemical transformation. *See supra* ¶61. Other compounds are added to facilitate the process but do not participate in the chemical transformation. *See supra* ¶61. Compound 4 and the claimed acid fall in the former category, reacting with each other and causing a chemical transformation of Compound 4 to the claimed PMO. The following chemical reaction schemes show how these compounds react directly to create a new compound.

Figure 13. Detritylation of Compound 4

79. Above, I show a trityl group, an exemplary protecting group disclosed in the '322 patent (Ex. 2 at 15:23), to illustrate a PMO protected at its 3'-nitrogen position (corresponding to Compound 4). This trityl group is removed by an acid (represented with "H+" in green), which reacts directly with the 3'-nitrogen in the PMO.

80. In other words, the direct reaction between Compound 4 and the acid chemically transforms Compound 4 to the PMO (**Figure 14**). Sarepta's interpretation of the claims reflects the nature of this reaction by clarifying that Compound 4 reacts directly with an acid.



**Figure 14**. Schematic Representation of Step f)

81. NS again disagrees with Sarepta's construction, arguing that a skilled artisan would have understood "directly" in Sarepta's construction to exclude the presence of other reagents or ingredients. As noted above, I am not aware of any chemistry articles or textbooks that support NS's position. *See supra* ¶64. Further, that is *not* how a skilled artisan would have understood "directly." Instead, a skilled artisan would have understood "directly" to mean that Compound 4 and an acid directly react with each another, causing the chemical transformation of Compound 4 to the PMO or oligomer. *See supra* ¶64; *see*, *e.g.*, Ex. 13 at 274-75.

### 3. NS's Construction Is Ambiguous

- 82. NS proposes interpreting step f) to mean "chemically reacting Compound 4 with an acid, in order to form the oligomer [or the PMO]." Similar to NS's construction of step e) above, NS's understanding of step f) is flawed.
- 83. NS's construction does not specify that Compound 4 used in step f) is produced in step e). NS's construction arguably allows using Compound 4 from other sources, or additional, unrecited steps between steps e) and f), even if those steps chemically transform Compound 4. NS's expansive construction is contrary to the claim language and the specification. In the case of claim 6, the claim instructs a skilled artisan to use "said Compound 4," referring back to Compound 4 formed in step e). See supra ¶13-15, 71. This is consistent with the specification, instructing a skilled artisan to use Compound 4 "produced in" step e). See supra ¶46-51, 71; Ex. 2 at 23:1-56. While claim 1 does not recite "said," a skilled artisan would have understood that Compound 4 made in step e) is used in step f) based on the overall structure of the claim, indicating that the recited steps should be performed in the specified order. See supra ¶72. The specification does not indicate that Compound 4 can be obtained from any source other than step e), further supporting this understanding. See Ex. 2 at 23:1-56.
- 84. NS's construction also does not specify that step f) must occur after step e). But in both claims 1 and 6, step f) is the final step of the claimed method. *See supra* ¶¶13-15. Thus, it is logical to read the claims to require performing step f) after step e). Consistent with this, the specification also lists step f) (corresponding to Step D in the specification) as the last step of PMO synthesis. *See* Ex. 2 at cols. 14-23; *see supra* ¶¶46-51, 72. Based on this claim language and description of the process in the specification, step f) must occur after step e).
- 85. NS's construction indicates that the reaction of step f) is performed "in order to form the oligomer [or the PMO]." Under NS's construction, it is unclear whether PMO formation

is just a goal of the process, as opposed to a result that must be achieved by the reaction. If it does not require actual PMO formation, NS's construction is contrary to the language of step f) that the reaction must "form the oligomer [or the PMO]." *See supra* ¶13-15, 71. Further, step f) is the final step of both claims 1 and 6, each directed to obtaining the oligomer (claim 1) or the PMO (claim 6). It is therefore logical that step f) results in the oligomer (claim 1) and the PMO (claim 6). The specification similarly explains that step f) (corresponding to Step D in the specification) "produce[s]" PMO. Ex. 2 at 23:1-56; *see supra* ¶46-51, 71.

86. NS's construction also fails to specify that Compound 4 and an acid must react directly. This omission potentially expands step f) to cover reactions that are not mentioned in the claims or specification. For example, under NS's construction, step f) could cover a multi-step reaction in which Compound 4 reacts with unrecited compound X, forming unrecited compound Y, which then reacts with an acid to form the PMO. This is not how a skilled artisan would have understood the reaction of step f), which states that Compound 4 reacts with the acid to form the PMO. *See supra* ¶77-81. This is also not how the specification describes step f), contemplating only the direct reaction between Compound 4 and an acid. *See supra* ¶¶46-51, 71; Ex. 2 at 23:1-56.

I declare under penalty of perjury that all statements made herein of my knowledge are true, and that all statements made herein on information and belief are believed to be true.

Date: February 5, 2023

Bradley L. Pentelute, Ph.D.

## Appendix A

### Curriculum Vitae

#### BRADLEY L. PENTELUTE

Professor, Department of Chemistry, MIT
Extramural Member, Koch Institute for Integrative Cancer Research, MIT
Associate Member, Broad Institute of MIT and Harvard
Member, Center for Environmental Health Sciences, MIT

77 Massachusetts Avenue, Room 18-596 Phone: (617) 324-0180 Cambridge, MA 02139 Email: blp@mit.edu

### <u>Degrees</u>:

Ph.D., Organic Chemistry, University of Chicago, 2008, Thesis Advisor: Steve Kent

M.S., Chemistry, University of Chicago, 2004

B.S., Chemistry, University of Southern California, 2003

B.A., Psychology, University of Southern California, 2003

### **Employment**:

Professor, Department of Chemistry, Massachusetts Institute of Technology, 2021–present

Associate Professor, Department of Chemistry, Massachusetts Institute of Technology, 2016–2021

Assistant Professor, Department of Chemistry, Massachusetts Institute of Technology, 2011–2016

Postdoctoral Fellow, Department of Microbiology and Molecular Genetics, Harvard Medical School, 2008–2011, John Collier's Lab

Senior Scientist, Ethos Pharmaceuticals, 1/2008–9/2008

Graduate Student, Department of Chemistry, University of Chicago, 2004–2008, Steve Kent's Lab

### **External Positions Held:**

Associate Editor, Scientific Reports, Nature, 2016–present

American Peptide Society, Nominating Committee, 2015–present

Visiting Professor, Tokyo Institute of Technology, Tokyo, Japan, 2019–2020

Visiting Professor, Osaka University, Osaka, Japan 2015–2018

NIH Ad Hoc Grant Reviewer, 2018

Committee member, Safety Culture in Academic Research Laboratories, The National Academies, 2013

Ad Hoc Grant Reviewer NSF Grants, 2015

1 of 18 - CV Pentelute

### **Honors**:

Rao Makineni Lectureship, American Peptide Society, 2021

Blavatnik Award Finalist, 2018

Eli Lilly Award in Biological Chemistry, 2018

Bristol-Myers Squibb Innovation Award, 2017

Amgen Young Investigator Award, 2016

Novartis Early Career Award in Organic Chemistry, 2015

Sloan Research Fellow in Chemistry, 2015

NSF CAREER Award, 2014

Sontag Distinguished Scientist Award, 2013

Young Chemical Biologist Award, International Chemical Biology Society, 2013

Damon Runyon-Rachleff Innovation Award, 2013

Vallee Foundation Travel Award, 2012

Collier Award, Gordon Conference, Microbial Toxins and Pathogenicity, 2010

Poster Prize, University of Chicago, Science at the Interface, 2008

Student Travel Award for Australian Peptide Society, 2006

U.S.C. Chemistry Alumni Award for Outstanding Undergraduate Research, 2003

Renaissance Scholar, U.S.C., 2003

### <u>Undergraduate Students Supervised:</u>

Bald, Ridings, 2021–present (MIT)

Berger, Tatiana, 2012–2013 (Boston University)

Buchwald, Nathan, 2016–2017 (Brown University)

Castro, Manuel, 2015

Chang, Richard, 2013–2015 (MIT)

Choo, Zi-Ning, 2013–2016 (MIT)

Dorminy, Sweet Tea, 2011 (MIT)

Gandhi, Dhyey, 2019–2020 (MIT)

Grupe, Hannah, 2019–present (MIT)

Kang, Hansol, 2012–2015 (MIT)

Kuborn, Thomas, Amgen Scholar, 2015 (U. of Wisconsin Oshkosh)

Lee, Yelim, 2016–2017 (Wellesley University)

Li, Xiuyuan, 2011–2013 (Stanford University)

Maina, Wacira, 2011

Policarpo, Rocco, 2011–2013 (Harvard University)

Sharygin, Daniel, 2022–present (MIT)

Ye, Tong, 2021 (MIT)

### Ph.D. and M.D./Ph.D. Students Supervised:

Antilla, Sarah An-ning (in progress)

Callahan, Alex Joseph (in progress)

Cho, Yehlin (in progress)

Cowfer, Amanda Elizabeth (in progress), NSF Graduate Research Fellowship

- Dai, Peng (Thesis: Site-Selective Modification of Cysteine Residues, 2018, Postdoctoral Researcher, Rice University)
- D'Angelo, Kyan (in progress, co-mentored with M. Movassaghi)
- Dieppa-Matos, Diomedes (in progress)
- Evans, Ethan Daniel (Thesis: Long Peptides for Cysteine Arylation, 2018, Postdoctoral Researcher, Alm Lab, MIT)
- Fadzen, Colin MacLaine (Thesis: Peptide-Mediated Delivery of Antisense Oligonucleotides and Chemotherapeutics Across Biological Barriers, 2018, M.D./Ph.D., Harvard Medical School)
- Farquhar, Charlotte Eleanor (in progress), NSF Graduate Research Fellowship
- Holden, Rebecca Lynn (Thesis: Addressing delivery and synthesis challenges for peptide-based cancer vaccines, 2020, Postdoctoral Researcher, Hacohen Lab, The Broad Institute of MIT and Harvard)
- Lee, Michael Alan (in progress)
- Loftis, Alexander Robert (Thesis: Re-targeting of anthrax toxin binding for immunomodulation and targeted cancer therapy, 2020, Life Sciences Specialist, L.E.K. Consulting)
- Lu, Zeyu (Thesis: Protective Antigen-mediated Delivery of Biomolecules, 2018, Equity Analyst, Goldman Sachs)
- Mallek, Aaron John (Thesis: Organometallic Palladium Reagents for Polypeptide Bioconjugation and Macrocyclization, 2021, Research Scientist, Angiex)
- Mijalis, Alexander James (Thesis: Automated Flow Peptide Synthesis, 2018, Postdoctoral Researcher, Church Lab, Harvard University)
- Mong, Surin Khai (Thesis: Investigation and Application of Heterochiral Proteins Enabled by Flow-Based Peptide Synthesis, 2017, Staff Scientist, Choate Hall & Stewart)
- Quartararo, Anthony James (Thesis: De novo discovery of synthetic peptide binders to protein-protein interfaces, 2020, Scientist I, FogPharma)
- Rabideau, Amy Ellen (Thesis: Delivery of Biomolecules into Mammalian Cells Using Anthrax Toxin, 2015, Senior Scientist, Moderna Therapeutics)
- Rodriguez, Jacob Joshua Lee (in progress, co-mentored with S. Buchwald), NSF Graduate Research Fellowship
- Rojas, Anthony (Thesis: Palladium Reagents for Bioconjugation, 2018, Assistant Professor, Salisbury University)
- Saebi, Azin (in progress, co-mentored with S. Buchwald), NSF Graduate Research Fellowship Santos, Michael Keith (Thesis: Protein engineering of targeted cancer therapies, 2017, Senior Software Developer, Athena Healthcare)
- Schissel, Carly Katherine (2022, Postdoctoral Researcher, Schepartz Lab, U.C. Berkeley)
- Simon, Mark David (Thesis: Fast Flow Biopolymer Synthesis, 2017, Scientist, Gingko Biosciences)
- Tuang, Suan (Thesis: Development of a reactive peptide sequence for site-selective bioconjugations, 2019, M.D./Ph.D., Harvard Medical School)
- Vinogradov, Alexander Alexandrovich (Thesis: New Methods for Synthesis and Modification of Peptides and Proteins, 2017, Postdoctoral Researcher, University of Tokyo)

Wolfe, Justin Mahoney (Thesis: Peptide Conjugation to Enhance Oligonucleotide Delivery, 2018, Senior Staff Scientist, Boston Children's Hospital)

Ye, Xiyun (in progress)

Zhang, Chi (Thesis: Cysteine Arylation, 2017, Postdoctoral Researcher, Boyden Lab, MIT)

### Postdoctoral Researchers Supervised:

Albin, John (2018–present)

Akcay, Gizem (2012–2013, Senior Scientist, AstraZeneca)

Bandyopadhyay, Anupam (2016–2018, Assistant Professor, Indian Institute of Technology Ropar, India)

Brown, Joseph (2019–present)

Buslov, Ivan (2017–2019, Senior Expert, SIBUR Chemicals, Moscow)

Cho, Choi-Fong (2014–2015, Assistant Professor in Neurosurgery, Harvard Medical School)

Cohen, Daniel Tzvi (2015–2017, Scientist, AbbVie)

Dhanjee, Heemal (2018–present, NIH Postdoctoral Fellow, co-mentored with S. Buchwald)

Evans, Ethan (2018, Postdoctoral Researcher, Alm Lab, MIT)

Gandhesiri, Satish (2021–present)

Gates, Zachary (2014–2020, Senior Scientist, p53Lab, Agency for Science, Technology and Research, Singapore)

Gazvoda, Martin (2020–2021, co-mentored with S. Buchwald, Assistant Professor, University of Ljubljana, Slovenia)

Grob, Nathalie (2020–present)

Hartrampf, Nina (2018–2019, Assistant Professor, University of Zurich)

Hanna, Cameron (2021–2022, Scientist II, Unnatural Products)

Ishoey, Mette (2016–2017, Assistant Professor, University of Copenhagen)

Jbarra, Muhammad (2019–2021, Assistant Professor, Tel Aviv University)

Khan, Kashif (2014–2018, Research Investigator II, Bristol-Myers Squibb)

Kubota, Koji (2016–2018, Assistant Professor, Hokkaido University, Japan)

Lautrette, Guillaume (2014–2016, Bioanalytical Monitor, SGS, France)

Li, Chengxi (2019–2021, Assistant Professor, Zhejiang University, China)

Liao, Xiaoli (2011–2014, Senior Scientist, AbbVie)

Lee, Hong Geun (2012–2016, Assistant Professor, Seoul University, South Korea)

Lee, Yen-Chun (2019–2021, Assistant Professor, National Cheng Kung University, Taiwan)

Lopez Vidal, Eva Maria (2018–2020, Scientist, Entrada Therapeutics)

Mijalis, Alexander (2018, Postdoctoral Researcher, Church Lab, Harvard University)

Miller, Edward (2021–present)

Pomplun, Sebastian (2019–2021, Assistant Professor, Leiden University)

Rondon, Aurélie (2021–present)

Rössler, Simon (2020–present, co-mentored with S. Buchwald)

Senter, Timothy (2015–2016, Senior Research Scientist, Vertex Pharmaceuticals)

Shugrue, Christopher (2019–2021, NIH Postdoctoral Fellow, Assistant Professor, University of Richmond)

Spokoyny, Alexander (2012–2014, Associate Professor, University of California, Los Angeles)

Tao, Jason (2018–2021, co-mentored with S. Buchwald)

Tan, Xuyu (2018–2021, Research Scientist, Janssen)

Totaro, Kyle (2014–2016, Scientist, Amide Technologies)

Touti, Faycal (2014–2018, Senior Scientist, Glympse Bio)

Truex, Nicholas (2018–present), NIH Postdoctoral Fellow

Vecchiarello, Nicholas (2021–present)

Wolfe, Justin (2018, Senior Staff Scientist, Boston Children's Hospital)

Wood, Thomas Melvin (2021–present)

Wong, Jeffrey Ying Kit (2021–present)

Yesilcimen, Ahmet (2022–present)

Zhang, Chi (2018–2019, Postdoctoral Researcher, Boyden Lab, MIT)

Zhang, Genwei (2019–present)

Zhang, Peiyuan (2021–present)

Zou, Yekui (2012–2013)

### Research Scientists Supervised:

Kitahara, Katsushi, Ph.D. (2021–present, Sumitomo Dainippon Pharma Co., Osaka, Japan)

Lampe, John, Ph.D. (2014, Associate Director, Epizyme)

Loas, Andrei, Ph.D. (2018–present)

Takeuchi, Hironori, Ph.D. (2021–present, Sumitomo Dainippon Pharma Co., Osaka, Japan)

### **Technical Associates Supervised:**

Hanna, Stephanie (2018–2021)

Halloran, Katie (2015–2016)

Lozano Salazar, Lia (2021–present)

Poskus, Mackenzie (2018–2020)

Wilson, Jessica (2015–2016)

### Visiting Researchers Supervised:

Charalampidou, Anna (M.Sc. Student, Technical University Darmstadt, Germany, 2022)

Chen, Pu-Guang (Ph.D. Student, Tsinghua University, China, 2018)

Cheng, Yiran (Ph.D. Student, Nankai University, China, 2017–2018)

Chinnapen, Daniel (Research Scientist, Children's Hospital, Boston, 2012–2016)

Dunkelmann, Daniel (Ph.D. Student, ETH, Switzerland, 2014–2015)

Halloran, Katie (Cambridge University, U.K., 2016–2017)

Han, Hyojun (Yonsei University, Korea, 2011–2012)

Hanna, Cameron (Ph.D. Student, University of Sydney, Australia, 2019)

Hauck, Patrick (M.Sc. Student, University of Applied Sciences and Arts Northwestern Switzerland, 2019–2020)

Hirata, Yuki (Kirin Japan Industry, Japan, 2015–2016)

Lee, Yen-Chun (Ph.D. Student, Max Planck Institute of Molecular Physiology, Germany, 2017–2018)

Liu, Shunying (Associate Professor, East China Normal University, China, 2018)

Maki, Yuta (Ph.D. Student, Kyoto University, Japan, 2014–2015)

Manbo, Akihiro (Ph.D. Student, Osaka University, Japan, 2017–2018)

Menichelli, Massimiliano (M.Sc. Student, ETH, 2016)

Misteli, Roman (M.Sc. Student, ETH, 2022)

Nakamura, Taichi (Ph.D. Student, Chuo University, Japan, 2016)

van Scheppingen, Daphne (Eindhoven University of Technology, Netherlands, 2012–2014)

Qian, Elaine (2017)

Sato, Kohei (Assistant Professor, Tokyo Institute of Technology, 2022)

Schmitt, Adeline (M.Sc. Student, University of Strasbourg, 2019)

Sementa, Deborah (University of Naples 'Federico II', Italy, 2016)

Shimada, Arisa (Ph.D. Student, Osaka University, Japan, 2017–2018)

Somsen, Bente (Ph.D. Student, Eindhoven University of Technology, Netherlands, 2018–2019)

Wang, Binyou (B.Sc. Student, Nankai University, China, 2018–2019)

Zuger, Vanessa (M.Sc. Student, ETH, 2017)

### **Teaching Experience:**

MIT 5.54, Frontiers in Chemical Biology, Fall 2012–2015

MIT 5.361 and 5.362, Biochemistry & Organic Lab, Spring 2012, 2013-present

MIT 5.383, Fast Flow Peptide and Protein Synthesis, 2015-present

MIT 5.111, Principles of Chemical Science (150 undergraduate students), 2018–present

MIT 5.07, Biochemistry first semester (40 undergraduate students), Fall 2019–present

### Service:

### Internal Service:

MIT Presidential Committee on Pre-health Advising, 2012–2014, 2019–present

(interview ~15 premed students for drafting recommendation letters, provide guidance, and coordinate application for medical school)

Improvement of MIT undergraduate chemistry modules, 2015–present

(raised money (2015 and 2019) to purchase new equipment for laboratories, designed and improved curriculum)

Chemistry Representative MIT institute faculty meetings, 2019–present

(attend all meetings and communicate important findings back to chemistry department)

MIT/Harvard M.D./Ph.D. committee member, 2016-present

(attend two meetings per year to discuss with M.D./Ph.D. students progress and challenges)

MIT Freshman Advisor, 2018–present

Faculty advisor ACS MIT Chemistry Club, 2012-present

(community magic shows, training new members, outreach)

Introduction to chemistry major, for MIT engineering students, 2017–present

Chemistry department, open house for incoming freshmen, faculty member, 2019–present

Chemistry department, open house, parents visiting weekend, magic shows, 2019–present

MIT Department Instrumentation committee member, 2014–present

Graduate Student Admission Committee Chair for Biological Chemistry, 2011–present

DOW-MIT Access Program in Chemistry, participant in visiting weekend, 2012–present

Chemistry Undergraduate Advisor (8 students), 2012-present

Thesis Chair (15 students), 2011–present

Faculty advisor, Chemistry career panel, 2012–2018

MIT Amgen Scholars graduate school admissions advisor, 2012–2017

External Service:

Associate Editor, Scientific Reports

Reviewer for Journal of the American Chemical Society, Nature Publishing Group, Chemical Science, Proceedings of the National Academy of Sciences of the United States of America, and ChemBioChem.

Ad-hoc Grant Reviewer, NIH SBCB and STTR, NSF CAREER

Guest Editor, ACS Chemical Reviews

### Publications from MIT (independent & collaborative):

- 1. Gazvoda, M.,\* Dhanjee, H.H.,\* Rodriguez, J., Brown, J.S., Farquhar, C.E., Truex, N.L., Loas, A., Buchwald, S.L.,\* Pentelute, B.L.\* (2022). Palladium-Mediated Incorporation of Carboranes into Small Molecules, Peptides, and Proteins. *Journal of the American Chemical Society*, 144(17):7852-7860 (\* = co-first authors, \* = co-corresponding authors).
- 2. Cho, C.F., Farquhar, C.E., Fadzen, C.M., Scott, B., Zhuang, P., von Spreckelsen, N., Loas, A., Hartrampf, N., Pentelute, B.L., Lawler, S.E. (2022). A Tumor-Homing Peptide Platform Enhances Drug Solubility, Improves Blood–Brain Barrier Permeability and Targets Glioblastoma. *Cancers*, 14(9):2207.
- 3. Schissel, C.K., Farquhar, C.E., Malmberg, A.B., Loas, A., Pentelute, B.L. (2022). Cell-Penetrating D-Peptides Retain Antisense Morpholino Oligomer Delivery Activity. *ACS Bio & Med Chem Au*, 1:150-160.
- 4. D'Angelo, K.A., Schissel, C.K., Pentelute, B.L., Movassaghi, M. (2022). Total synthesis of himastatin. *Science*, 375(6583):894-899.
- 5. Li, C., Callahan, A.J., Phadke, K.S., Bellaire, B., Farquhar, C.E., Zhang, G., Schissel, C.K., Mijalis, A.J., Hartrampf, N., Loas, A., Verhoeven, D.E., Pentelute, B.L. (2022). Automated Flow Synthesis of Peptide–PNA Conjugates. *ACS Central Science*, 8(2):205-213.
- 6. Yang, N.J., Isensee, J., Neel, D.V., Quadros, A.U., Zhang, H.B., Lauzadis, J., Liu, S.M., Shiers, S., Belu, A., Palan, S., Marlin, S., Maignel, J., Kennedy-Curran, A., Tong, V.S., Moayeri, M., Röderer, P., Nitzsche, A., Lu, M., Pentelute, B.L., Brüstle, O., Tripathi, V., Foster, K.A., Price, T.J., Collier, R.J., Leppla, S.H., Puopolo, M., Bean, B.P., Cunha, T.M., Hucho, T., Chiu, I.M. (2022). Anthrax toxins regulate pain signaling and can deliver molecular cargoes into ANTXR2<sup>+</sup> DRG sensory neurons. *Nature Neuroscience*, 25(2):168-179.
- 7. Zhang, G.,\* Brown, J.S.,\* Quartararo, A.J., Li, C., Tan, X., Hanna, S., Antilla, S., Cowfer, A.E., Loas, A., Pentelute, B.L. (2022). Rapid *de novo* discovery of peptidomimetic affinity reagents for human angiotensin converting enzyme 2. *Communications Chemistry*, 5:8 (\* = co-first authors).
- 8. López-Vidal, E.M.,\* Schissel, C.K.,\* Mohapatra, S., Bellovoda, K., Wu, C.L., Wood, J.A., Malmberg, A.B., Loas, A., Gómez-Bombarelli, R.,\* Pentelute, B.L.\* (2021). Deep Learning

- Enables Discovery of a Short Nuclear Targeting Peptide for Efficient Delivery of Antisense Oligomers. JACS Au, 1(11):2009-2020 (\* = co-first authors, # = co-corresponding authors).
- 9. Pomplun, S.,\* Jbara, M.,\* Schissel, C.K., Wilson Hawken, S., Boija, A., Li, C., Klein, I., Pentelute, B.L. (2021). Parallel Automated Flow Synthesis of Covalent Protein Complexes That Can Inhibit MYC-Driven Transcription. *ACS Central Science*, 7(8):1408-1418 (\* = cofirst authors).
- 10. Zhang, G., Li, C., Quartararo, A.J., Loas, A., Pentelute, B.L. (2021). Automated affinity selection for rapid discovery of peptide binders. *Chemical Science*, 12(32):10817-10824.
- 11. Loftis, A.R.,\* Zhang, G.,\* Backlund, C., Quartararo, A.J., Pishesha, N., Hanna, C.C., Schissel, C.K., Garafola, D., Loas, A., Collier, R.J., Ploegh, H., Irvine, D.J., Pentelute, B.L. (2021). An in vivo selection-derived d-peptide for engineering erythrocyte-binding antigens that promote immune tolerance. *Proceedings of the National Academy of Sciences of the United States of America*, 118(34):e2101596118 (\* = co-first authors).
- 12. Schissel, C.K.,\* Mohapatra, S.,\* Wolfe, J.M., Fadzen, C.M., Bellovoda, K., Wu, C.L., Wood, J.A., Malmberg, A.B., Loas, A., Gómez-Bombarelli, R.,\* Pentelute, B.L.\* (2021). Deep learning to design nuclear-targeting abiotic miniproteins. *Nature Chemistry*, 13(10):992-1000 (\* = co-first authors, \* = co-corresponding authors).
- 13. Jbara, M.,\* Pomplun, S.,\* Schissel, C.K., Hawken, S.W., Boija, A., Klein, I., Rodriguez, J., Buchwald, S.L., Pentelute, B.L. (2021). Engineering Bioactive Dimeric Transcription Factor Analogs via Palladium Rebound Reagents. *Journal of the American Chemical Society*, 143(30):11788-11798 (\* = co-first authors).
- 14. Li, C.,\* Callahan, A.J.,\* Simon, M.D., Totaro, K.A., Mijalis, A.J., Phadke, K.S., Zhang, G., Hartrampf, N., Schissel, C.K., Zhou, M., Zong, H., Hanson, G.J., Loas, A., Pohl, N.L.B., Verhoeven, D.E., Pentelute, B.L. (2021). Fully automated fast-flow synthesis of antisense phosphorodiamidate morpholino oligomers. *Nature Communications*, 12(1):4396 (\* = co-first authors).
- 15. Mallek, A.J., Pentelute, B.L., Buchwald, S.L. (2021). Selective *N*-Arylation of p-Aminophenylalanine in Unprotected Peptides with Organometallic Palladium Reagents. *Angewandte Chemie International Edition*, 60(31):16928-16931 (# = co-corresponding authors).
- 16. Jbara, M., Rodriguez, J., Dhanjee, H.H., Loas, A., Buchwald, S.L., Pentelute, B.L. (2021). Oligonucleotide Bioconjugation with Bifunctional Palladium Reagents. *Angewandte Chemie International Edition*, 60(21):12109-12115 (# = co-corresponding authors).
- 17. Van Egeren, D.,\* Novokhodko, A.,\* Stoddard, M.,\* Tran, U., Zetter, B., Rogers, M., Pentelute, B.L., Carlson, J.M., Hixon, M., Joseph-McCarthy, D., Chakravarty, A. (2021). Risk of rapid evolutionary escape from biomedical interventions targeting SARS-CoV-2 spike protein. *PLoS ONE*, 16(4):e0250780 (\* = co-first authors).
- 18. Von Spreckelsen, N., Fadzen, C.M., Hartrampf, N., Ghotmi, Y., Wolfe, J.M., Dubey, S., Yang, B.Y., Kijewski, M.F., Wang, S., Farquhar, C., Bergmann, S., Zdioruk, M., Wasserburg, J.R., Scott, B., Murrell, E., Bononi, F.C., Luyt, L.G., DiCarli, M., Lamfers, M.L.M., Ligon, K.L., Chiocca, E.A., Viapiano, M.S., Pentelute, B.L., Lawler, S.E., Cho, C.-

- F.# (2021). Targeting Glioblastoma Using a Novel Peptide Specific to a Deglycosylated Isoform of Brevican. *Advanced Therapeutics*, 4(4):2000244 (# = co-corresponding authors).
- 19. Tuang, S.,\* Dieppa-Matos, D.,\* Zhang, C., Shugrue, C.R., Dai, P., Loas, A., Pentelute, B.L. (2021). A reactive peptide interface for site-selective cysteine bioconjugation. *Chemical Communications*, 57(26):3227-3230 (\* = co-first authors).
- 20. Lu, Z.,\* Truex, N.L.,\* Melo, M.B., Cheng, Y., Li, N., Irvine, D.J., Pentelute, B.L. (2021). IgG-Engineered Protective Antigen for Cytosolic Delivery of Proteins into Cancer Cells. *ACS Central Science*, 7(2):365-378 (\* = co-first authors).
- 21. Hu, Z.,\* Leet, D.E.,\* Allesøe, R.L.,\* Oliveira, G., Li, S., Luoma, A.M., Liu, J., Forman, J., Huang, T., Iorgulescu, J.B., Holden, R., Sarkizova, S., Gohil, S.H., Redd, R.A., Sun, J., Elagina, L., Giobbie-Hurder, A., Zhang, W., Peter, L., Ciantra, Z., Rodig, S., Olive, O., Shetty, K., Pyrdol, J., Uduman, M., Lee, P.C., Bachireddy, P., Buchbinder, E.I., Yoon, C.H., Neuberg, D., Pentelute, B.L., Hacohen, N., Livak, K.J., Shukla, S.A., Olsen, L.R., Barouch, D.H., Wucherpfennig, K.W., Fritsch, E.F., Keskin, D.B., Wu, C.J., Ott, P.A. (2021). Personal neoantigen vaccines induce persistent memory T cell responses and epitope spreading in patients with melanoma. *Nature Medicine*, 27(3):515-525 (\* = co-first authors).
- 22. Longwell, C.K., Hanna, S., Hartrampf, N., Sperberg, R.A.P., Huang, P.S., Pentelute, B.L., Cochran, J.R. (2021). Identification of N-Terminally Diversified GLP-1R Agonists Using Saturation Mutagenesis and Chemical Design. *ACS Chemical Biology*, 16(1):58-66.
- 23. Pomplun, S., Jbara, M., Quartararo, A.J., Zhang, G., Brown, J.S., Lee, Y.-C., Ye, X., Hanna, S., Pentelute, B.L. (2021). De Novo Discovery of High Affinity Peptide Binders for the SARS-CoV-2 Spike Protein. *ACS Central Science*, 7(1):156-163.
- 24. Dhanjee, H.H.,\* Buslov, I.,\* Windsor, I.A., Raines, R.T., Buchwald, S.L.,\* Pentelute, B.L.\* (2020). Palladium—Protein Oxidative Addition Complexes by Amine-Selective Acylation. *Journal of the American Chemical Society*, 142(51):21237-21242 (\* = co-first authors, \* = co-corresponding authors).
- 25. Pomplun, S., Gates, Z.P., Zhang, G., Quartararo, A.J., Pentelute, B.L. (2020). Discovery of Nucleic Acid Binding Molecules from Combinatorial Biohybrid Nucleobase Peptide Libraries. *Journal of the American Chemical Society*, 142(46):19642-19651.
- 26. Mohapatra, S.,\* Hartrampf, N.,\* Poskus, M., Loas, A., Gomez-Bombarelli, R.,\* Pentelute, B.L.\* (2020). Deep Learning for Prediction and Optimization of Fast-Flow Peptide Synthesis. *ACS Central Science*, 6(12):2277-2286 (\* = co-first authors, \* = co-corresponding authors).
- 27. Quartararo, A.J., Gates, Z.P., Somsen, B.A., Hartrampf, N., Ye, X., Shimada, A., Kajihara, Y., Ottmann, C., Pentelute, B.L. (2020). Ultra-large chemical libraries for the discovery of high-affinity peptide binders. *Nature Communications*, 11(1):3183.
- 28. Fadzen, C.M., Wolfe, J.M., Zhou, W., Cho, C.-F., von Spreckelsen, N., Hutchinson, K.T., Lee, Y.-C., Chiocca, E.A., Lawler, S., Yilmaz, O.H., Lippard, S.J., Pentelute, B.L. (2020). A Platinum(IV) Prodrug-Perfluoroaryl Macrocyclic Peptide Conjugate Enhances Platinum Uptake in the Brain. *Journal of Medicinal Chemistry*, 63(13):6741-6747 (# = co-corresponding authors).
- 29. Lu, Z.,\* Paolella, B.R.,\* Truex, N.L.,\* Loftis, A.R., Liao, X., Rabideau, A.E., Brown, M.S., Busanovich, J., Beroukhim, R.,\* Pentelute, B.L.\* (2020). Targeting Cancer Gene

- Dependencies with Anthrax-Mediated Delivery of Peptide Nucleic Acids. *ACS Chemical Biology*, 15(6):1358-1369 (\* = co-first authors, # = co-corresponding authors).
- 30. Pomplun, S.,\* Shugrue, C.R.,\* Schmitt, A.M., Schissel, C.K., Farquhar, C.E., Pentelute, B.L. (2020). Secondary Amino Alcohols: Traceless Cleavable Linkers for Use in Affinity Capture and Release. *Angewandte Chemie International Edition*, 59(28):11566-11572 (\* = co-first authors).
- 31. Lindemann, W.R., Mijalis, A.J., Alonso, J.L., Borbat, P.P., Freed, J.H., Arnaout, M.A., Pentelute, B.L., Ortony, J.H. (2020). Conformational dynamics in extended RGD-containing peptides. *Biomacromolecules*, 21(7):2786-2794.
- 32. Loftis, A.R.,\* Santos, M.S.,\* Truex, N.L., Biancucci, M., Satchell, K.J.F., Pentelute, B.L. (2020). Anthrax protective antigen retargeted with single-chain variable fragments delivers enzymes to pancreatic cancer cells. *ChemBioChem*, 21(19):2772-2776 (\* = co-first authors).
- 33. Hartrampf, N., Saebi, A., Poskus, M., Gates, Z.P., Callahan, A.J., Cowfer, A.E., Hanna, S., Antilla, S., Schissel, C.K., Quartararo, A.J., Ye, X., Mijalis, A.J., Simon, M.D., Loas, A., Liu, S., Jessen, C., Nielsen, T.E., Pentelute, B.L. (2020). Synthesis of proteins by automated flow chemistry. *Science*, 368(6494):980-987.
- 34. Dhanjee, H.H.,\* Saebi, A.,\* Buslov, I., Loftis, A.R., Buchwald, S.L.,\* Pentelute, B.L.\* (2020). Protein-Protein Cross-Coupling via Palladium-Protein Oxidative Addition Complexes from Cysteine Residues. *Journal of the American Chemical Society*, 142(20): 9124-9129 (\* = co-first authors, \* = co-corresponding authors).
- 35. Albin, J.S., Pentelute, B.L. (2020). Efficient flow synthesis of human antimicrobial peptides. *Australian Journal of Chemistry*, 73(4):380-388.
- 36. Loas, A., Pentelute, B.L. (2020). Introduction: Peptide Chemistry. *Chemical Reviews*, 120(6):3049-3050.
- 37. Mandala, V.S., Loftis, A.R., Shcherbakov, A.A., Pentelute, B.L., Hong, M. (2020). Atomic structures of closed and open influenza B M2 proton channel reveal the conduction mechanism. *Nature Structural and Molecular Biology*, 27(2):160-167.
- 38. Lindemann, W.R., Evans, E.D., Mijalis, A.J., Saouaf, O.M., Pentelute, B.L., Ortony, J.H. (2020). Quantifying residue-specific conformational dynamics of a highly reactive 29-mer peptide. *Scientific Reports*, 10(1):2597.
- 39. Jack, S., Madhivanan, K., Ramadesikan, S., Subramanian, S., Edwards, D.F., Elzey, B.D., Dhawan, D., McCluskey, A., Kischuk, E.M., Loftis, A.R., Truex, N., Santos, M., Lu, M., Rabideau, A., Pentelute, B.L., Collier, J., Kaimakliotis, H., Koch, M., Ratliff, T.L., Knapp, D.W., Aguilar, R.C. (2020). A novel, safe, fast and efficient treatment for Her2-positive and negative bladder cancer utilizing an EGF-anthrax toxin chimera. *International Journal of Cancer*, 146(2):449-460.
- 40. Truex, N.L., Holden, R.L., Wang, B.Y., Chen, P.-G., Hanna, S., Hu, Z., Shetty, K., Olive, O., Neuberg, D., Hacohen, N., Keskin, D.B., Ott, P.A., Wu, C.J.,\* Pentelute, B.L.\* (2020). Automated Flow Synthesis of Tumor Neoantigen Peptides for Personalized Immunotherapy. *Scientific Reports*, 10(1):723 (\* = co-corresponding authors).

- 41. Fadzen, C.M.,\* Holden, R.L.,\* Wolfe, J.M.,\* Choo, Z.-N., Schissel, C., Yao, M., Hanson, G.J., Pentelute, B.L. (2019). Chimeras of Cell-Penetrating Peptides Demonstrate Synergistic Improvement in Antisense Efficacy. *Biochemistry*, 58(38):3980-3989 (\* = co-first authors).
- 42. Wang, B., Dai, P., Ding, D., Del Rosario, A., Grant, R.A., Pentelute, B.L., Laub, M.T. (2019). Affinity-based Capture and Identification of Protein Effectors of the Growth Regulator ppGpp. *Nature Chemical Biology*, 15(2):141-150.
- 43. Romano, K.P., Warrier, T., Poulsen, B.E., Nguyen, P.H., Loftis, A.R., Saebi, A., Pentelute, B.L., Hung, D.T. (2019). Mutations in *pmrB* Confer Cross-Resistance between the LptD Inhibitor POL7080 and Colistin in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 63(9):e00511-19.
- 44. Zhang, C., Vinogradova, E.V., Spokoyny, A.M., Buchwald, S.B., Pentelute, B.L. (2019). Arylation Chemistry for Bioconjugation. *Angewandte Chemie International Edition*, 58(15):4810-4839.
- 45. Evans, E.D., Gates, Z.P., Sun, Z.-Y.J., Mijalis, A.J., Pentelute, B.L. (2019). Conformational Stabilization and Rapid Labeling of a 29-Residue Peptide by a Small Molecule Reaction Partner. *Biochemistry*, 58(10):1343-1353.
- 46. Evans, E.D., Pentelute, B.L. (2019). Studies on a landscape of perfluoroaromatic-reactive peptides. *Organic and Biomolecular Chemistry*, 17(7):1862-1868.
- 47. Cohen, D.T., Zhang, C., Fadzen, C.M., Mijalis, A.J., Hie, L., Johnson, K.D., Shriver, Z., Plante, O., Miller, S.J., Buchwald, S.B., Pentelute, B.L. (2019). A Chemoselective Strategy for Late Stage Functionalization of Complex Small Molecules with Polypeptides and Proteins. *Nature Chemistry*, 11(1):78-85.
- 48. Touti, F., Gates, Z.P., Bandyopadhyay, A., Lautrette, G., Pentelute, B.L. (2019). In-solution enrichment identifies peptide inhibitors of protein-protein interactions. *Nature Chemical Biology*, 15(4):410-418.
- 49. Touti, F.,\* Lautrette, G.,\* Johnson, K., Delaney, J., Wollacott, A., Tissire, H., Viswanathan, K., Shriver, Z., Mong, S., Mijalis, A.J., Plante, O.J., Pentelute, B.L. (2018). Antibody-Bactericidal Macrocyclic Peptide Conjugates to Target Gram-Negative Bacteria. *ChemBioChem*, 19(19):2039-2044 (\* = co-first authors).
- 50. Bergmann, S., Lawler, S.E., Qu, Y., Fadzen, C.M., Wolfe, J.M., Regan, M.S., Pentelute, B.L., Agar, N.Y.R., Cho, C.-F. (2018). Blood–brain-barrier organoids for investigating the permeability of CNS therapeutics. *Nature Protocols*, 13(12):2827-2843.
- 51. Gates, Z.P., Vinogradov, A.A., Quartararo, A.J., Bandyopadhyay, A., Choo, Z.N., Evans, E.D., Halloran, K.H., Mijalis, A.J., Mong, S.K., Simon, M.D., Standley, E.A., Styduhar, E.D., Tasker, S.Z., Touti, F., Weber, J.M., Jamison, T.F., Pentelute, B.L. (2018). Xenoprotein engineering via synthetic libraries. *Proceedings of the National Academy of Sciences of the United States of America*, 115(23):E5298-E5306.
- 52. Dunkelmann, D.L., Hirata, Y., Totaro, K.A., Cohen, Z., Gates, Z.P., Pentelute, B.L. (2018). Amide-forming chemical ligation via O-acyl hydroxamic acids. *Proceedings of the National Academy of Sciences of the United States of America*, 115(15):3752-3757.
- 53. Belashov, I.A., Crawford, D.W., Cavender, C.E., Dai, P., Beardslee, P.C., Mathews, D.H., Pentelute, B.L., McNaughton, B.R., Wedekind, J.E. (2018). Structure of HIV TAR in

- complex with a Lab-Evolved RRM provides insight into duplex RNA recognition and synthesis of a constrained peptide that impairs transcription. *Nucleic Acids Research*, 46(13):6401-6415.
- 54. Garcia-Castillo, M.D., Chinnapen, D.J., Te Welscher, Y.M., Gonzalez, R.J., Softic, S., Pacheco, M., Mrsny, R.J., Kahn, C.R., von Andrian, U.H., Lau, J., Pentelute, B.L., Lencer, W.I. (2018). Mucosal absorption of therapeutic peptides by harnessing the endogenous sorting of glycosphingolipids. *eLife*, 7:e34469.
- 55. Machen, A.J., O'Neil, P.T., Pentelute, B.L., Villar, M.T., Artigues, A., Fisher, M.T. (2018). Analyzing Dynamic Protein Complexes Assembled On and Released From Biolayer Interferometry Biosensor Using Mass Spectrometry and Electron Microscopy. *Journal of Visualized Experiments*, (138):e57902.
- 56. Moynihan, K.D., Holden, R.L., Mehta, N.K., Wang, C., Karver, M.R., Dinter, J., Liang, S., Abraham, W., Melo, M.B., Zhang, A.Q., Li, N., Gall, S.L., Pentelute, B.L., Irvine, D.J. (2018). Enhancement of Peptide Vaccine Immunogenicity by Increasing Lymphatic Drainage and Boosting Serum Stability. *Cancer Immunology Research*, 6(9):1025-1038.
- 57. Hartmann, S., Lopez Cruz, R., Alameh, S., Ho, C.C., Rabideau, A., Pentelute, B.L., Bradley, K.A., Martchenko, M. (2018). Characterization of Novel Piperidine-Based Inhibitor of Cathepsin B-Dependent Bacterial Toxins and Viruses. *ACS Infectious Diseases*, 4(8):1235-1245.
- 58. Slough, D.P., McHugh, S.M., Cummings, A.E., Dai, P., Pentelute, B.L., Kritzer, J.A., Lin, Y.S. (2018). Designing Well-Structured Cyclic Pentapeptides Based on Sequence-Structure Relationships. *Journal of Physical Chemistry B*, 122(14):3908-3919.
- 59. Zhang, C.,\* Dai, P.,\* Vinogradov, A.A., Gates, Z.P., Pentelute, B.L. (2018). Site-Selective Cysteine-Cyclooctyne Conjugation. *Angewandte Chemie International Edition*, 57(22):6459-6463 (\* = co-first authors).
- 60. Wolfe, J.M.,\* Fadzen, C.M.,\* Holden, R.L., Yao, M. Hanson, G.J., Pentelute, B.L. (2018). Perfluoroaryl Bicyclic Cell-Penetrating Peptides for Delivery of Antisense Oligonucleotides. *Angewandte Chemie International Edition*, 57(17):4756-4759 (\* = co-first authors).
- 61. Wolfe, J.M.,\* Fadzen, C.M.,\* Choo, Z-.N., Holden, R.L., Yao, M., Hanson, G.J., Pentelute, B.L. (2018). Machine Learning to Predict Cell-Penetrating Peptides for Antisense Delivery. *ACS Central Science*, 4(4):512-520 (\* = co-first authors).
- 62. Kubota, K., Dai, P., Pentelute, B.L., Buchwald, S.L. (2018). Palladium Oxidative Addition for Peptide and Protein Cross-linking. *Journal of the American Chemical Society*, 140(8):3128-3133.
- 63. Evans, E.D., Pentelute, B.L. (2018). Discovery of a 29-Amino-Acid Reactive Abiotic Peptide for Selective Cysteine Arylation. *ACS Chemical Biology*, 13(3):527-532.
- 64. Dai, P., Williams, J.K., Zhang, C., Welborn, M., Shepherd, J.J., Zhu, T., van Voorhis, T., Hong, M., Pentelute, B.L. (2017). A structural and mechanistic study of Π-clamp-mediated cysteine perfluoroarylation. *Scientific Reports*, 7:7954.
- 65. Elkins, M.R., Williams, J.K., Gelenter, M.D., Dai, P., Kwon, B., Sergeyev, I.V., Pentelute, B.L., Hong, M. (2017). Cholesterol-binding site of the influenza M2 protein in lipid bilayers

- from solid-state NMR. *Proceedings of the National Academy of Sciences of the United States of America*, 114(49):12946-12951.
- 66. Fadzen, C.M.,\* Wolfe, J.M.,\* Cho, C.F., Chiocca, E.A., Lawler, S.E., Pentelute, B.L. (2017). Perfluoroarene-Based Peptide Macrocycles to Enhance Penetration Across the Blood-Brain Barrier. *Journal of the American Chemical Society*, 139(44):15628-15631 (\* = co-first authors).
- 67. Mong, S.K., Cochran, F.V., Yu, H., Graziano, Z., Lin, Y., Cochran, J.R., Pentelute, B.L. (2017). Heterochiral Knottin Protein: Folding and Solution Structure. *Biochemistry*, 56(43):5720-5725.
- 68. Vinogradov, A., Gates, Z.P., Zhang, C., Quartararo, A.J., Halloran, K.H., Pentelute, B.L. (2017). Library Design-Facilitated High-Throughput Sequencing of Synthetic Peptide Libraries. *ACS Combinatorial Science*, 19(11):694-701.
- 69. Rojas, A., Pentelute, B.L., Buchwald, S.L. (2017). Water-Soluble Palladium Reagents for Cysteine S-Arylation under Ambient Aqueous Conditions. *Organic Letters*, 19(16):4263-4266.
- 70. Cho, F., Wolfe, J.M., Fadzen, C.M., Calligaris, D., Hornburg, K., Chiocca, E.A., Agar, N.Y.R., Pentelute, B.L., Lawler, S.E. (2017). Blood-brain barrier spheroids as an in vitro screening platform for brain-penetrating agents. *Nature Communications*, 8:15623.
- 71. Biancucci, M., Rabideau, A.E., Lu, Z., Loftis, A.R., Pentelute, B.L., Satchell, K.J.F. (2017). Substrate recognition of MARTX Ras/Rap1-specific endopeptidase. *Biochemistry*, 56(21):2747-2757.
- 72. Mijalis, A.,\* Thomas, D.A.,\* Simon, M.D., Adamo, A., Beaumont, R., Jensen, K.F., Pentelute, B.L. (2017). A fully automated flow-based approach for accelerated peptide synthesis. *Nature Chemical Biology*, 13(5):464-466 (\* = co-first authors).
- 73. Rojas, A.J., Zhang, C., Vinogradova, E.V., Buchwald, N., Reilly, J., Pentelute, B.L.,\* Buchwald, S.B.\* (2017). Divergent unprotected peptide macrocyclization by palladium-mediated cysteine arylation. *Chemical Science*, 8(6):4257-4262 (\* = co-corresponding authors).
- 74. Lee, H.G., Lautrette, G., Pentelute, B.L.,\* Buchwald, S.B.\* (2017). Palladium-mediated arylation of lysine in unprotected peptides. *Angewandte Chemie International Edition*, 56(12):3177-3181 (\* = co-corresponding authors).
- 75. Pentelute, B.L., Wang, L. (2016). Editorial overview: Chemistry for biopolymers to investigate and even move beyond nature. *Current Opinion in Chemical Biology*, 34:v-vi.
- 76. Simon, M.D., Maki, Y., Vinogradov, A.A., Zhang, C., Yu, H., Lin, Y., Kajihara, Y., Pentelute, B.L. (2016). D-amino acid scan of two small proteins. *Journal of the American Chemical Society*, 138(7):12099-12111.
- 77. Dai, P., Zhang, C., Welborn, M., Shepherd, J., Zhu, T., Van Voorhis, T., Pentelute, B.L. (2016). Salt effect accelerates site-selective cysteine bioconjugation. *ACS Central Science*, 2(9):637-646.
- 78. Lautrette, G., Touti, F., Lee, H.G., Dai, P., Pentelute, B.L. (2016). Nitrogen arylation for macrocyclization of unprotected peptides. *Journal of the American Chemical Society*, 138(27):8340-8343.

- 79. Rabideau, A.E., Pentelute, B.L. (2016). Delivery of non-native cargo into mammalian cells using anthrax lethal toxin. *ACS Chemical Biology*, 11(6):1490-1501.
- 80. Totaro, K.A., Liao, X., Bhattacharya, K., Finneman, J.L., Sperry, J.B., Massa, M.A., Thorn, J., Ho, S.V., Pentelute, B.L. (2016). Systematic investigation of EDC/sNHS-mediated bioconjugation reactions of carboxylated peptide substrates. *Bioconjugate Chemistry*, 27(4):994-1004.
- 81. Luhmann, T.,\* Mong, S.K.,\* Simon, M.D., Meinel, L., Pentelute, B.L. (2016). A perfluoroaromatic abiotic analogue of H2 relaxin enabled by rapid flow-based peptide synthesis. *Organic and Biomolecular Chemistry*, 14(13):3345-3349 (\* = co-first authors).
- 82. Vinogradov, A.A., Choo, Z.-N., Totaro, K.A., Pentelute, B.L. (2016). Macrocyclization of unprotected peptide isocyanates. *Organic Letters*, 18(6):1226-1229.
- 83. Vinogradov, A.A., Simon, M.D., Pentelute, B.L. (2016). C-terminal modification of fully unprotected peptide hydrazides via in situ generation of isocyanates. *Organic Letters*, 18(6):1222-1225.
- 84. Zhang, C., Welborn, M., Zhu, T., Santos, M., Yang, N., Van Voorhis, T., Pentelute, B.L. (2016). π-Clamp mediated cysteine conjugation. *Nature Chemistry*, 8(2):120-128.
- 85. Rabideau, A., Pentelute, B.L. (2015). A D-Amino Acid at the N-Terminus of a Protein Abrogates Its Degradation by the N-End Rule Pathway. *ACS Central Science*, 1(8):423-430.
- 86. Vinogradova, E.V.,\* Zhang, C.,\* Spokoyny, A.M., Pentelute, B.L.,\* Buchwald, S.L.\* (2015). Organometallic palladium reagents for cysteine bioconjugation. *Nature*, 526(7575):687-691 (\* = co-first authors, \* = co-corresponding authors).
- 87. Cohen, D.T.,\* Zhang, C.,\* Pentelute, B.L.,\* Buchwald, S.L.\* (2015). An Umpolung Approach for the Chemoselective Arylation of Selenocysteine in Unprotected Peptides. *Journal of the American Chemical Society*, 137(31):9784-7 (\* = co-first authors, \* = co-corresponding authors).
- 88. Rabideau, A.,\* Liao, X.,\* Akcay, G., Pentelute, B.L. (2015). Translocation of Non-Canonical Polypeptides into Cells Using Protective Antigen. *Scientific Reports*, 5:11944 (\* = co-first authors).
- 89. Jensen, J., Pentelute, B.L., Collier, R.J., Zhou, H. (2015). Atomic structure of anthrax PA pore elucidates toxin translocation. *Nature*, 521(7553):545-549.
- 90. Vinogradov, A.A., Evans, E.D., Pentelute, B.L. (2015). Total synthesis and biochemical characterization of mirror image barnase. *Chemical Science*, 6(5):2997-3002.
- 91. Liao, X.,\* Rabideau, A.,\* Pentelute, B.L. (2015). Delivery of mirror image polypeptides into cells. *Chemical Science*, 6(1):648-653 (\* = co-first authors).
- 92. Zhang, C., Dai, P., Spokoyny, A., Pentelute, B.L. (2014). Enzyme catalyzed macrocyclization of long unprotected peptides. *Organic Letters*, 16(14):3652-3655.
- 93. Liao, X.,\* Rabideau, A.,\* Pentelute, B.L. (2014). Delivery of antibody mimics into mammalian cells via anthrax toxin protective antigen. *ChemBioChem*, 15(16):2458-2466 (\* = co-first authors).
- 94. Policarpo, R.L., Kang, H., Liao, X., Rabideau, A.E., Simon, M.D., Pentelute, B.L. (2014). Flow-Based Enzymatic Ligation via Sortase A. *Angewandte Chemie International Edition*, 53(35):9203-9208.

- 95. Mong, S.K., Vinogradov, A.A., Simon, M.D., Pentelute, B.L. (2014). Rapid Total Synthesis of DARPin pE59 and Barnase. *ChemBioChem*, 15(5):721-733.
- 96. Simon, M.D., Heider, P., Adamo, A., Vinogradov, A.A., Mong, S.K., Li, X., Berger, T., Policarpo, R.P., Zhang, C., Zou, Y., Liao, X., Spokoyny, A.M., Jensen, K.F., Pentelute, B.L. (2014). Rapid Flow-Based Peptide Synthesis. *ChemBioChem*, 15(5):713-720.
- 97. Zou, Y., Spokoyny, A.M., Zhang, C., Simon, M.D., Yu, H., Lin, Y., Pentelute, B.L. (2014). Convergent Diversity-Oriented Side-Chain Macrocyclization Scan for Unprotected Polypeptides. *Organic and Biomolecular Chemistry*, 12(4):566-573.
- 98. Zhang, C., Spokoyny, A.M., Zou, Y., Simon, M.D., Pentelute, B.L. (2013). Enzymatic "click" ligation: selective cysteine modification enabled by promiscuous glutathione Stransferase. *Angewandte Chemie International Edition*, 52(52):14001-14005.
- 99. Spokoyny, A., Zou, Y., Ling, J., Pentelute, B.L. (2013). A perfluoroaryl-cysteine S<sub>N</sub>Ar chemistry approach to unprotected peptide stapling. *Journal of the American Chemical Society*, 135(16):5946-5949.
- 100. Hubbard, B.P., Gomes, A.P., Dai, H., Li, J., Case, W.A., Considine, T., Riera, T.V., Lee, J.L., E, S.Y., Lamming, D.W., Pentelute, B. L., Schuman, E.R., Stevens, L.A., Ling, A.J.Y., Armour, S.M., Michan, S., Zhao, H., Jiang, Y., Sweitzer, S.M., Blum, C.A., Disch, J.S., Ng, P.Y., Howitz, K.T., Rolo, A.P., Hamuro, Y., Moss, J., Perni, R.B., Ellis, J.L., Vlasuk, G.P., Sinclair, D.A. (2013). Evidence for a common mechanism of SIRT1 regulation by allosteric activators. *Science*, 339(6124):1216-1219.
- 101. Ling, J.J., Policarpo, R.L., Rabideau, A.E., Liao, X., Pentelute, B.L. (2012). Protein thioester synthesis enabled by sortase. *Journal of the American Chemical Society*, 134(26):10749-10752.

### <u>Publications prior to MIT (\* = from graduate school)</u>:

- 102. Akkaladevi, N., Mukherejee, S., Katayama, H., Janowiak, B., Patel, D., Gogol, E.P., Pentelute, B.L., Collier, R.J., Fisher, M.T. (2015). Following Nature's Lead: On the Construction of Membrane-Inserted Toxins in Lipid Bilayer Nanodiscs. *Journal of Membrane Biology*, 248(3):595-607.
- \*Bunker, R.D., Mandal, K., Chaston, J.J., Pentelute, B.L., Lott, J.S., Kent, S.B., Baker, E.N. (2015). A functional role of Rv1738 in mycobacterium tuberculosis persistence suggested by racemic protein crystallography. *Proceedings of the National Academy of Sciences of the United States of America*, 112(14):4310-4315.
- 104. Akkaladevi, N., Hinton-Chollet, L., Katayama, H., Mitchell, J., Szeersen, L., Mukherjee, S., Gogol, E.P., Pentelute, B.L., Collier, R.J., Fisher, M.T. (2013). Assembly of anthrax toxin pore: lethal-factor complexes into lipid nanodiscs. *Protein Science*, 22(4):492-501.
- 105. Naik, S., Brock, S., Akkaladevi, N., Tally, J., McGinn-Straub, W., Zhang, N., Gao, P., Gogol, E.P., Pentelute, B.L., Collier, R.J., Fisher, M.T. (2013). Monitoring the kinetics of the pH-driven transition of the anthrax toxin prepore to the pore by biolayer interferometry and surface plasmon resonance. *Biochemistry*, 52(37): 6335-6347.
- 106. Gogol, E.P., Akkaladevi, N., Szerszen, L., Mukherjee, S., Chollet-Hinton, L., Pentelute, B.L., Collier, R.J., Fisher, M.T. (2013). Three dimensional structure of the anthrax toxin

- translocon-lethal factor complex by cryo-electron microscopy. *Protein Science*, 22(5):586-594.
- 107. \*Kent, S., Sohma, Y., Liu, S., Bang, D., Pentelute, B.L., Mandal, K. (2012). Through the looking glass a new world of proteins enabled by chemical synthesis. *Journal of Peptide Science*, 18(7):428-436.
- 108. \*Mandal, K., Pentelute, B.L., Bang, D., Gates, Z.P., Torbeev, V.Y., Kent, S.B.H. (2012). Design, total synthesis, and X-ray structure of a protein having a novel linear-loop polypeptide chain topology. *Angewandte Chemie International Edition*, 51(6):1481-1486.
- 109. \*Sawaya, M.R., Pentelute, B.L., Kent, S.B., Yeates, T.O. (2012). Single-wavelength phasing strategy for quasi-racemic protein diffraction data. *Acta Crystallografica D*, 68(1):62-68.
- 110. \*Liu, S., Pentelute, B.L., Kent, S.B. (2012). Convergent Chemical Synthesis of [Lysine<sup>24,38,83</sup>] Human Erythropoietin. *Angewandte Chemie International Edition*, 51(4):993-999.
- 111. Fischer, A., Holden, M., Pentelute, B.L., Collier, R.J. (2011). Ultrasensitive detection of anthrax toxin protein translocation across droplet hydrogel bilayers. *Proceedings of the National Academy of Sciences of the United States of America*, 108(40):16577-16581.
- 112. Lee J., Kwon Y., Pentelute, B.L., Bang, D. (2011). Use of Model Peptide Reactions for the Characterization of a Kinetically Controlled Ligation (KCL). *Bioconjugate Chemistry*, 22(8):1645-1649.
- 113. Pentelute, B.L., Sharma, O., Collier, R.J. (2011). Chemical dissection of protein translocation through anthrax toxin pore. *Angewandte Chemie International Edition*, 50(10):2294-2296.
- 114. \*Pentelute, B.L., Gates, Z.P., Mandal, K., Sawaya, M.R., Yeates, T.O., Kent, S.B. (2010). Total chemical synthesis and X-ray structure of kaliotoxin by racemic protein crystallography. *Chemical Communications*, 46(43):8174-8176.
- 115. Pentelute, B.L., Barker, A.P., Janowiak, B.E., Kent, S.B., Collier, R.J. (2010). A semisynthesis platform for investigating structure-function relationships in the N-terminal domain of the anthrax Lethal Factor. *ACS Chemical Biology*, 5(4):359-364.
- 116. \*Mandal, K., Pentelute, B.L., Tereshko, V., Thammavaongsa, V., Kossiakoff, A.A., Kent, S.B. (2009). Total chemical synthesis and racemic protein crystallography used to determine the X-ray structure of plectasin by direct methods. *Protein Science*, 18(6):1146-1154.
- 117. \*Mandal, K., Pentelute, B.L., Tereshko, V., Kossiakoff, A.A., Kent, S.B. (2009). X-ray structure of native scorpion toxin BmBKTx1 by racemic protein crystallography using direct methods. *Journal of the American Chemical Society*, 131 (4): 1362-1363.
- 118. \*Pentelute, B.L., Gates, Z.P., Tereshko, V., Dashnau, J.L, Vanderkooi, J.M., Kossiakoff, A.A., Kent, S.B. (2008). X-ray structure of snow flea antifreeze protein determined by racemic crystallization of synthetic protein enantiomers. *Journal of the American Chemical Society*, 130(30): 9695-9701.
- 119. \*Pentelute, B.L., Gates, Z.P., Tereshko, V., Dashnau, J.L, Vanderkooi, J.M., Kossiakoff, A.A., Kent, S.B. (2008). Mirror image forms of snow flea antifreeze protein prepared by total

- chemical synthesis have identical antifreeze activities. *Journal of the American Chemical Society*, 130(30): 9702-9707.
- 120. \*Sohma, Y., Pentelute, B.L., Whittaker, J., Qin-xin, H., Whittaker, L.J., Weiss, M.A., Kent, S.B. (2008). Total chemical synthesis of Insulin-like Growth Factor I (IGF-1) and [Gly7D-Ala]IGF-1 by use of native chemical ligation. *Angewandte Chemie International Edition*, 47(6):1102-1106.
- 121. \*Johnson, E.C.B., Malito, E., Shen, Y., Pentelute, B.L., Rich, D., Florián, J., Wei-Jen, T., Kent, S.B. (2007). Insights from atomic-resolution X-ray structures of chemically-synthesized HIV-1 protease in complex with inhibitors. *Journal of Molecular Biology*, 373(3):573-586.
- \*Pentelute, B.L., Kent, S.B. (2007). Selective desulfurization of cysteine in the presence of Cys(Acm) in polypeptides obtained by native chemical ligation. *Organic Letters*, 9(4):687-690.
- \*Bang, D., Pentelute, B.L., Kent, S.B. (2006). Kinetically controlled ligation for the convergent chemical synthesis of proteins. *Angewandte Chemie International Edition*, 45(24): 3985-3988.
- \*Bang, D., Pentelute, B.L., Gates, Z.P., Kent, S.B. (2006). Direct on-resin synthesis of peptide-(alpha)thiophenylesters for use in native chemical ligation. *Organic Letters*, 8(6):1049-1052.

### Patents and Patent Applications:

- 1. S.B.H. Kent, B.L. Pentelute, D. Bang, E. Johnson, T. Durek. Convergent synthesis of proteins by kinetically controlled ligation. U.S. Pat. 7,674,881 (March 9, 2010).
- 2. R.J. Collier, B.L. Pentelute. Method for delivering agents into cells using bacterial toxins. U.S. Pat. 9,079,952 (July 14, 2015), U.S. Pat. 9,850,475 (December 26, 2017).
- 3. R.J. Collier, I. Chiu, B.L. Pentelute. Compositions and methods for treatment of pain. U.S. Pat. Appl. 20180251740A1 (September 6, 2018).
- 4. R.J. Collier, A. McCluskey, B. L. Pentelute. Methods for making targeted protein toxins by Sortase-mediated protein ligation. U.S. Pat. Appl. 20160102332A1 (April 14, 2016).
- 5. G. Marsischky, B.L. Pentelute, R.J. Collier, A. McCluskey. Methods for targeted modification of genomic DNA. U.S. Pat. Appl. 20170198307A1 (July 23, 2017).
- 6. B.L. Pentelute, L. Xiaoli, A.E. Rabideau, J. Ling, R.L. Policarpo. Protein retrosplicing enabled by a double ligation reaction. U.S. Pat. 9,731,029 (August 15, 2017).
- 7. L. Xiaoli, A.E. Rabideau, B.L. Pentelute, J. Ling, G. Akcay, R.J. Collier. Translocation of non-natural chemical entities through anthrax protective antigen pore. U.S. Pat. 9,498,538 (November 22, 2016).
- 8. B.L. Pentelute, A.M. Spokoyny, Y. Zou, C. Zhang. Modification of peptides via  $S_NAr$  reactions of thiols with fluorinated aromatics. U.S. Pat. 9,018,172 (April 28, 2015).
- 9. M.D. Simon, B.L. Pentelute, A. Adamo, P.L. Heider, K.F. Jensen. Solid phase peptide synthesis processes and associated systems. U.S. Pat. 9,868,759 (January 16, 2018), U.S. Pat. 9,695,214 (July 4, 2017), U.S. Pat. 9,169,287 (October 27, 2015).
- 10. B.L. Pentelute, C. Zhang. Cysteine arylation directed by a genetically encodable pi-clamp. U.S. Pat. 9,181,297 (November 10, 2015).

- 11. S.L. Buchwald, B.L. Pentelute, A. Spokoyny, E. Vinogradova, C. Zhang. Transition metal-based selective functionalization of chalcogens in biomolecules. U.S. Pat. Appl. 20190055280A1 (February 21, 2019).
- 12. D.A. Thomas III, A. Mijalis, M.D. Simon, B.L. Pentelute, S. Mong. Methods and systems for solid phase peptide synthesis. U.S. Pat. Appl. 20210047365A1 (February 18, 2021).
- 13. S.L. Buchwald, B.L. Pentelute, D.T. Cohen, C. Zhang. Selective arylation of dichalcogenides in biomolecules. U. S. Pat. 10,117,948 (November 6, 2018).
- 14. B.L. Pentelute, F. Touti. Solution-phase affinity selection of inhibitors from combinatorial peptide libraries. U.S. Pat. Appl. 20190300576A1 (October 3, 2019).
- 15. K.D. Moynihan, R.L. Holden, D.J. Irvine, B.L. Pentelute. Albumin binding peptide conjugates and methods thereof. U.S. Pat. Appl. 20190046654A1 (February 14, 2019).
- 16. B.L. Pentelute, E.D. Evans. Self-labeling miniproteins and conjugates comprising them. U.S. Provisional Appl. 62/450,127.
- 17. J.M. Wolfe, C.M. Fadzen, Z.-N. Choo, R.L. Holden, M. Yao, G.J. Hanson, B.L. Pentelute. Cell-penetrating peptides for antisense delivery. U.S. Pat. Appl. 20200316210A1 (October 8, 2020).
- 18. J.M. Wolfe, C.M. Fadzen, R.L. Holden, M. Yao, G.J. Hanson, B.L. Pentelute. Bicyclic peptide oligonucleotide conjugates. U.S. Pat. Appl. 20200237922A1 (July 30, 2020).
- 19. K.A. Totaro, M.D. Simon, M. Zhou, H. Zong, G.J. Hanson, B.L. Pentelute. Processes for preparing phosphorodiamidate morpholino oligomers via fast-flow synthesis. U.S. Pat. Appl. 20200362339A1 (November 19, 2020).
- 20. B.L. Pentelute, C.M. Fadzen, R.L. Holden, J.M. Wolfe, Z.-N. Choo, M. Yao, G.J. Hanson. Chimeric peptides for antisense delivery. U.S. Pat. Appl. 20210260206A1 (August 26, 2021).
- 21. J.M. Wolfe, C.M. Fadzen, B.L. Pentelute, G.J. Hanson. Trimeric peptides for antisense delivery. International Pat. Appl. WO2020028254A1 (February 6, 2020).
- 22. Z. Lu, B.L. Pentelute. Functionalization of bacterial effector translocase protein by chemical conjugation. U.S. Pat. Appl. 20200061201A1 (February 27, 2020).
- 23. S.J. Lippard, B.L. Pentelute, O. Omer, J.M. Wolfe, C.M. Fadzen, W. Zhou. Platinum prodrug perfluoroaryl peptide conjugates. U.S. Pat. Appl. 20210023129A1 (January 28, 2021).
- 24. C.K. Schissel, S. Mohapatra, J.M. Wolfe, C.M. Fadzen, C.-L. Wu, A.B. Malmberg, G.J. Hanson, B.L. Pentelute, R. Gomez-Bombarelli, E.M. Lopez Vidal. Designing antisense oligonucleotide delivery peptides by interpretable machine learning. International Pat. Appl. WO2021150867A1 (July 29, 2021).

### Companies started:

Co-founder, Amide Technologies, Cambridge, MA, 2018-present

Co-scientific founder, Resolute Biosciences, Cambridge, MA, 2018-present

Co-founder, Tegrigen Therapeutics, Cambridge, MA, 2021-present

Co-scientific founder, New Frontier Bio, Cambridge, MA, 2021-present

## Appendix B

### **List of Materials Considered**

Exhibit Number	Description
2	U.S. Patent No. 10,683,322
4	Alberts et al., <i>Molecular Biology of the Cell</i> 191-234, 299-374 (4th ed. 2002) ("Alberts 2002")
5	Chan et al., "Antisense Oligonucleotides: From Design to Therapeutic Application," <i>Clin. Exp. Pharmacol. Physiol.</i> (2006) 33(5-6): 533-540 ("Chan 2006")
6	Moulton et al., "Gene Knockdowns in Adult Animals: PPMOs and Vivo- Morpholinos," <i>Molecules</i> (2009) 14(3): 1304-1323 ("Moulton 2009")
7	Pon, "Solid-Phase Supports for Oligonucleotide Synthesis," <i>Curr. Protoc. Nucleic Acid Chem.</i> (2000) 00(1): 3.1.13.1.28 ("Pon 2000")
8	Summerton et al., "Morpholino Antisense Oligomers: Design, Preparation, and Properties," <i>Antisense Nucleic Acid Drug Dev.</i> (1997) 7(3): 187-195 ("Summerton 1997")
9	Summerton, "Morpholinos and PNAs Compared," Lett. Pept. Sci. (2003) 10: 215-236 ("Summerton 2003")
10	Collins English Dictionary 1443 (11th ed. 2011)
13	Mase et al., Comprehensive Organic Synthesis 273-339 (2d ed. 2014)

# EXHIBIT 15

### IN THE UNITED STATES DISTRICT COURT DISTRICT OF DELAWARE

NIPPON SHINYAKU CO., LTD., Plaintiff,	
v.	)
SAREPTA THERAPEUTICS, INC., Defendant.	C.A. No. 21-1015 (GBW)  (C.A. No. 21-1015 (GBW)
SAREPTA THERAPEUTICS, INC., Defendant and Counter-Plaintiff	) ) ) )
v.	)
NIPPON SHINYAKU CO., LTD. and NS PHARMA, INC., Plaintiff and Counter-Defendants.	) ) ) )

### **DECLARATION OF NATHAN W. LUEDTKE, PH.D**

### I, Nathan W. Luedtke, declare as follows:

1. I have been asked by outside counsel for Nippon Shinyaku Co., Ltd. and NS Pharma, Inc. (collectively "NS") to offer my opinions relating to certain claim construction disputes between the parties in connection with the above-referenced matter. I have been informed that this case has involved the following Asserted Patents, and that the parties dispute the meaning of certain terms used in those Asserted Patents:

No.	U.S. Patent No.
1	9,708,361 ("'361 Patent")
2	10,385,092 ("'092 Patent")
3	10,407,461 ("'461 Patent")
4	10,487,106 ("'106 Patent")
5	10,647,741 ("'741 Patent")
6	10,662,217 ("'217 Patent")
7	10,683,322 ("'322 Patent")
8	9,994,851 ("'851 Patent")
9	10,227,590 ("'590 Patent")

### 10 10,266,827 ("'827 Patent")

I have been informed and understand that the first seven patents listed (the "NS Patents") have been asserted by Nippon Shinyaku against Sarepta, and that the latter three patents listed (the "UWA Patents") have been asserted by Sarepta against NS.

2. I submit this Declaration to provide relevant background information regarding the technology at issue in the Asserted Patents, and to set forth my opinion about the meaning of certain disputed terms of the NS Patents. If called as a witness to testify at a claim construction hearing, I expect to testify on the following topics and provide opinions and testimony on what is summarized in this declaration.

### I. QUALIFICATIONS, EXPERIENCE, AND PUBLICATIONS

- 3. The following is a brief summary of my background and qualifications. My background and qualifications are more fully set out in my curriculum vitae ("CV"), attached as Exhibit 1 to this Declaration.
- 4. In 1997, I earned a Bachelors of Science in Biochemistry at the University of Washington (Seattle, Washington). By 1999, I had completed my Masters of Science in Chemistry at the University of California (San Diego, California). I also completed my Ph.D in Chemistry at the University of California (San Diego, California) in 2003. I underwent my postdoctoral training as a National Institutes of Health (NIH) Postdoctoral Fellow in chemical biology at Yale University from 2003-2006.
- 5. In 2006, I became an Assistant Professor in the Institute of Organic Chemistry at the University of Zurich. I was appointed to a tenure-track position for the chair of Prof. Hans-Jurgen Hansen and served as an Assistant Professor until 2012. I was then granted tenure in the Department of Chemistry at the University of Zurich for research and teaching in organic chemistry and chemical biology and was promoted to Associate Professor in 2012. In 2018, I was

made Full Professor in the Department of Chemistry at the University of Zurich and was named the Endowed Chair of Organic Chemistry and Chemical Biology. Since 2019, I have worked at McGill University as a Full Professor in the Department of Chemistry.

- 6. Throughout my career, my research has focused on the synthesis of modified nucleosides, nucleotides, and oligonucleotides. My work has sought to develop new methods for making modified nucleic acids for analytical and therapeutic applications.
- 7. As my CV shows, I have roughly 70 peer-reviewed publications in this or related fields since 1996, with many additional invited lectures. I am also named as an inventor or co-inventor on numerous U.S. and international patents and/or patent applications related to the synthesis and modification of nucleic acids. As a result, I have first-hand experience in solid-phase synthesis of oligomeric nucleotides using commercially available systems including the Milligen Cyclone Plus, Applied Biosystems 3400, and MerMade 4 as well as oligopeptides on the Symphony® automated solid-phase synthesizer.
- 8. I have received awards and recognition for my work, both internally within my research institution and externally from other organizations, including numerous awarded grants. Notably, in 2022, I was awarded the James McGill Professorship Award which recognizes a senior scholar's status as an outstanding and original researcher of world-class caliber and an international leader in his or her field.
- 9. I am also active in various professional organizations in this field. I am an appointed member of WORLD.MINDS and the Centre de recherche en biologie structural (CRBS). I have been an appointed editorial board member of various journals, including Signal Transduction and Targeted Therapy, Molecules, and Chimia (the journal of the Swiss Chemical Society).

10. Throughout my career, I have taken an interest in teaching and mentoring the next generation of scientists. Beyond regularly teaching coursework in biotechnology, organic chemistry, bioorganic and advanced nucleic acids chemistry, I have supervised and mentored dozens of post-doctoral fellows and research staff, as well as Ph.D, MSc, and undergraduate students who have gone on to take positions in industry conducting oligonucleotide synthesis.

### II. COMPENSATION

I am being compensated for my time spent on this matter at a rate of \$695 per hour for depositions and testimony and \$595 per hour for other work, plus reasonable expenses. My compensation is not related to the outcome of this action, and I have no financial interest in the outcome of this case.

### III. MATERIALS CONSIDERED

- 12. In preparing this declaration, I have considered the materials identified in this declaration, as well as the following materials:
  - the '322 Patent;
  - the parties' Joint Claim Construction Chart (December 15, 2022, D.I. 144) and accompanying appendix;
  - NS's Opening Claim Construction Brief regarding the NS Patents;
  - Sarepta's Answering Claim Construction Brief regarding the NS Patents;
  - The Declaration of Dr. Bradley L. Pentelute; and
  - Any additional materials identified below.
- 13. I have also relied upon my decades of general experience in the field, though the testimony I offer is from the perspective of a person of ordinary skill as I have defined it below.

### IV. THE LAW OF CLAIM CONSTRUCTION

- 14. This section describes my understanding of currently applicable legal principles based on my conversations with counsel, which I have used in forming the opinions set forth herein. I have used my understanding of these legal principles, as set forth in this section, to analyze the Asserted Patents. However, I am not a legal expert, and I do not offer legal opinions in this declaration.
- 15. I have been informed that claim construction is an issue of law for the Court to decide, although a particular claim construction may involve underlying factual issues that the Court must evaluate in reaching its determination. I am also informed that the construction of claims is a way of interpreting the claim language in order to understand and explain, but not to change, the scope of the claims.
- 16. I am informed that claim construction begins with a focus on the contents of the claim themselves and that the claims are generally given their ordinary and customary meaning. The ordinary and customary meaning is the meaning that the claim would have to a person of ordinary skill in the art in question at the time of the invention. I have been informed that a person of ordinary skill in the art is presumed to have read the patent's specification and prosecution history to better understand the context of the invention. I am also informed that in determining the ordinary meaning of a claim term, one should consult the intrinsic evidence, *e.g.*, the claims themselves (including chemical structures in the claims), the associated specifications and examples, and the prosecution history, and to a lesser extent, extrinsic evidence, such as dictionaries, treatises, and expert testimony.
- 17. I am also informed that where a patent derives from a parent application, such that they share the same written descriptions and use many common terms, the prosecution history of that parent application may be relevant to construing the patent at issue.

### V. PERSON OF ORDINARY SKILL IN THE ART ("POSA")

- 18. I understand from counsel that assessing the level of ordinary skill in an art may involve considering various factors, including the type of problems encountered in the art, prior art solutions to those problems, the rapidity with which innovations are made, the sophistication of the technology, and the educational level of active workers in the field.
  - 19. Dr. Pentelute and Sarepta have offered slightly differing definitions of a POSA.
- 20. I understand that Dr. Pentelute identified a POSA in the '322 Patent as someone with the following background:

a Ph.D. in chemistry, biochemistry, cell biology, genetics, molecular biology, or an equivalent, and several years of experience with antisense oligonucleotides for inducing exon skipping, who: (1) also would have been familiar with methods for making and testing the safety and efficacy of antisense oligonucleotides, both in vitro and in vivo, and the use of antisense oligonucleotides for inducing exon skipping in the context of medical conditions, such as DMD, that may be treated by administering antisense oligonucleotides and (2) would have had knowledge of and experience with chemical modifications that may be incorporated into antisense oligonucleotides, such as modifications to the backbone and/or nucleobases of the antisense oligonucleotides, and the potential impact of those modifications on the utility of the antisense oligonucleotides.

Ex. 14 (Pentelute Declaration) at ¶ 21.

21. In its Answering Brief, Sarepta provided a slightly different definition of a POSA:

Sarepta proposes that a skilled artisan would have had a Ph.D in chemistry, biochemistry, cell biology, genetics, molecular biology, or an equivalent, and several years of experience with antisense oligonucleotides for inducing exon skipping.

Ans. Br. at 2.

- 22. Based on my review of the '322 Patent and experience in the field, I disagree somewhat with the definition offered by Dr. Pentalute and Sarepta. Although I agree that a Ph.D with relevant experience would qualify as a POSA, I do not see why an M.S. or B.S./B.A. would not also qualify if they had relevant experience. Moreover, the '322 Patent is directed to a solid phase synthesis method. I disagree that specific experience with the use of antisense oligonucleotides for inducing exon skipping and/or testing the safety and efficacy of oligonucleotides is needed.
- 23. Instead, I propose that a POSA in the '322 Patent would have had a Ph.D or lower degree in chemistry, biochemistry, cell biology, genetics, molecular biology, or an equivalent, and several years of experience in the synthesis of oligomers, with additional education requiring less experience and vice versa. Nonetheless, I have considered the issues below under both my definition and Sarepta/Dr. Pentalute's definition and reach the same opinions under both.

### VI. DISPUTED TERMS

24. I understand the parties have disputes regarding the meaning of the following terms used in the Asserted Claims of the NS Patents:

Term	Claim Term/Issue
1	"antisense oligomer consisting of the nucleotide sequence of SEQ ID NO: 57"
2	"e) reacting said Compound 3 with a deprotecting agent to form Compound 4"
3	"f) reacting Compound 4 with an acid to form said oligomer" or "f) reacting said Compound 4 with an acid to form said PMO"

25. For this declaration, I have been asked to provide (and herein provide) expert analysis of Terms 2 and 3 but reserve my right to provide analysis on the parties' remaining

disputes as needed, for example, in response to any argument or expert opinion(s) offered by Sarepta.

26. As an initial note, Terms 2 and 3 are included as steps e) and f) of claims 1 and 6 of the '322 Patent. A full recitation of claims 1 and 6 is set forth below:

1. A solid-phase method of making an oligomer comprising a

phosphorodiamidate morpholino oligomer (PMO) and a group at the 5' end of said PMO, wherein said PMO

is 100% complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in a human dystrophin pre-mRNA,

wherein the 53rd exon in said human dystrophin pre-mRNA consists of a nucleotide sequence corresponding to SEQ ID NO: 1,

wherein said PMO hybridizes to said human dystrophin pre-mRNA with Watson-Crick base pairing,

wherein the phosphorodiamidate morpholino monomers of said PMO have the formula:

wherein each of R2 and R3 represents a methyl; and

wherein Base is a nucleobase selected from the group consisting of: uracil, cytosine, thymine, adenine, and guanine; and 6. A solid-phase method of making a

phosphorodiamidate morpholino oligomer (PMO) that

is 100% complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in a human dystrophin pre-mRNA,

wherein the 53rd exon in said human dystrophin pre-mRNA consists of a nucleotide sequence corresponding to SEQ ID NO: 1,

wherein said PMO hybridizes to said human dystrophin pre-mRNA with Watson-Crick base pairing,

wherein the phosphorodiamidate morpholino monomers of said PMO have the formula:

wherein each of R2 and R3 represents a methyl; and

wherein Base is a nucleobase selected from the group consisting of: uracil, cytosine, thymine, adenine, and guanine; and wherein the group at the 5' end of said PMO has the formula:

said method comprising:

a) providing Compound 1:

|Compound I|

| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
| Compound I|
|

wherein T represents trityl, monomethoxytrityl, or dimethoxytrityl; wherein each of R2 and R3 represents a methyl; and wherein BP is a protected Base,

b) reacting said Compound 1 with an acid to form Compound 2:

- c) reacting said Compound 2 with a morpholino monomer in the presence of a base and a solvent;
- d) repeating steps b) and c) until Compound 3 is complete:

wherein the 5' end of said PMO has the formula:

said method comprising:

a) providing Compound 1:

wherein T represents trityl, monomethoxytrityl, or dimethoxytrityl; wherein each of R2 and R3 represents a methyl; and wherein BP is a protected Base,

b) reacting said Compound 1 with an acid to form Compound 2:

- c) reacting said Compound 2 with a morpholino monomer in the presence of a base and a solvent;
- d) repeating steps b) and c) until Compound 3 is complete:

## e) reacting said Compound 3 with a deprotecting agent to form Compound 4:

$$R_{2}$$
 $R_{3}$ 
 $R_{3}$ 
 $R_{4}$ 
 $R_{5}$ 
 $R_{5$ 

## f) reacting Compound 4 with an acid to form said oligomer.

## e) reacting said Compound 3 with a deprotecting agent to form Compound 4:

### f) reacting said Compound 4 with an acid to form said PMO:

- 27. As is seen above, claims 1 and 6 are very similar and have a nearly identical structure. The solid-phase method of making set forth in claims 1 and 6 includes six steps: beginning with step a) and ending with step f).
- 28. Claims 1 and 6 also refer to several named Compounds: Compound 1, Compound 2, Compound 3, and Compound 4. When each of the Compounds is introduced in claims 1 and 6, they are expressly defined using the chemical structure of the Compound. "providing Compound 1:"

"to form Compound 2:"

[Compound 2]

$$R_2$$
 $N-P-O$ 
 $R_3$ 
 $N$ 
 $R_4$ 
 $R_5$ 
 $R_7$ 
 $R_8$ 
 $R_8$ 
 $R_8$ 
 $R_9$ 
 $R_9$ 

"until Compound 3 is complete:"

[Compound 3]

"to form Compound 4:"

- 29. After each Compound has been defined, claims 1 and 6 typically reference each Compound as "said Compound." I understand that in the context of a patent the word "said" is used to "refer[] back to an earlier phrase in the claim" and that when the word "said" is used, "the scope of the term will be the same as the scope of the earlier language." *See* Ex. 14 at f.n. 2. Or, in other words, the subsequent references to "said Compound" mean that the Compound should be given the same definition as was previously provided in the claim.
- 30. Since each Compound is defined by its chemical structure, a POSA would have understood each reference to "said Compound" to mean a Compound having the previously defined chemical structure.
- 31. Returning to the layout of the claim, the solid-phase method of synthesis can be broken into two main parts: steps a) d) describe building the oligomer, and steps e) and f) describe polishing or finishing steps that are performed to purify and/or finalize the oligomer.

- 32. In general, I agree that a POSA would have understood that the steps of building the oligomer must be performed in the listed order: step a) must be first, followed by step b), step c), and finally step d). However, a POSA would have understood that reversible chemical reactions could take place between each claimed step. For example, after step b) in which Compound 2 is formed, a POSA could perform a protonation reaction to obtain a compound that is slightly different than Compound 2—and would not meet its defined structure. The POSA could then reverse that reaction and reform Compound 2. The POSA could then continue with step c) using Compound 2 that was created during this intermediate reaction—not the exact Compound 2 that was created during step b).
- 33. While I agree that a POSA would have understood that the steps of building the oligomer in steps a) through d) must be performed in the listed order, it is my opinion that a POSA would have understood that steps e) and f) could be performed in any order. These polishing or finishing steps are dependent on the purification methods available to the POSA. A POSA would have understood that a variety of different purification schemes could be used, and that the order of steps e) and f) is not relevant to the claimed method.

VII. Term 2 – "e) reacting said Compound 3 with a deprotecting agent to form Compound 4"

34. The parties' proposed constructions for disputed Term 2 are shown below:

<u>Term</u>	NS's Position	Sarepta's Position
"e) reacting said	Plain and ordinary meaning - i.e.,	Plain and ordinary meaning, i.e.,
Compound 3 with a	chemically reacting Compound 3	chemically reacting a deprotecting
deprotecting agent to	with a deprotecting agent, in order	agent directly with Compound 3
form Compound 4"	to form Compound 4	of step d), which results in
('322 Patent Claims 1 and 6)		Compound 4.

- 35. In my opinion, a POSA would have understood Term 2 to mean "chemically reacting Compound 3 with a deprotecting agent, in order to form Compound 4" as is consistent with NS's proposed construction. I also note that replacing "in order to form Compound 4" in NS's proposed construction with either "to form Compound 4" or "which results in Compound 4" would also be consistent with how a POSA understood Term 2.
- 36. A POSA would have understood that Term 2 is not consistent with Sarepta's proposed construction for at least two reasons: (1) Sarepta's proposed construction adds the word "directly" to describe the chemical reaction, and (2) Sarepta's proposed construction requires that step e) use the specific Compound 3 that was the result "of step d)."
- 37. First, I will address the addition of the word "directly" in Sarepta's proposed construction.
- 38. As Dr. Pentalute describes, chemical reactions can involve both reagents (which are compounds that interact with each other to transform the chemical structures of those compounds), and other non-reagent compounds like solvents or catalysts (compounds that do not participate in the chemical transformation but facilitate the process). *See* Ex. 14 ¶ 61.
- 39. Sarepta argues that Term 2 requires Compound 3 and the deprotecting agent to be the only reagents in the reaction described in Term 2 and that other ingredients can only be used if they are non-reactive:

Further, as Dr. Pentelute explains, this is *not* how a skilled artisan would understand "directly." Pentelute Decl. ¶64; *see* Ex. 13 at 274-75. In a chemical reaction, some chemical compounds are directly involved in the reaction (e.g., Compound 3 and the deprotecting agent recited in step e)), whereas others may not chemically react but are added to facilitate the reaction (e.g., a solvent for diluting the deprotecting agent). Thus, contrary to NS's argument, "directly" does not exclude other reagents or ingredients used to facilitate the reaction between the recited compounds, and therefore is consistent with the claimed methods "comprising" step e).

Ans. Br. at 16.

- 40. Sarepta's position is wrong. Nothing in the claim language excludes the use of additional, unlisted reagents as part of the reaction of step e). Term 2 specifically uses the word "reacting," which a POSA would understand is not limited to only "direct reactions" between the two listed reagents. Indeed, a POSA would understand that certain reactions necessarily involve more than two reagents.
- 41. For example, consider the '322 Patent's disclosures related to step b) of claims 1 and 6. Step b) contains nearly identical language and structure as step e):
  - b) reacting said Compound 1 with an acid to form Compound 2;
  - e) reacting said Compound 3 with a deprotecting agent to form Compound 4; and

'322 Patent at cl. 1.

- 42. Given the similarity of the language, a POSA would understand that if step e) required Compound 3 to react "directly" with a deprotecting agent to form Compound 4 (as Sarepta proposes in its construction), step b) would require Compound 1 to react "directly" with an acid to form Compound 2. But the examples and disclosures in the '322 Patent demonstrate that this is not the case.
- 43. With respect to step b), the '322 Patent describes the general process as reacting Compound 1 with an acid to form Compound 2. *See e.g.*, '322 Patent at Col. 14:45-17:6. However, the '322 Patent provides more specifics regarding the acid—including the fact that it can be used in the form of a dilution:

This step can be performed by reacting Compound (11) with an acid.

The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

*Id.* at Col. 16:21-29.

The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

Id. at Col. 16:42-48.

44. The '322 Patent also specifies that after the acid reaction, a second neutralization reaction may be required:

After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, disopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

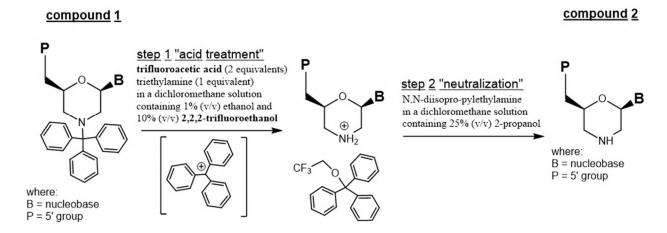
*Id.* at Col. 16:57-62.

45. Example 1 of the '322 Patent describes the specific acid "deblocking solution" and base "neutralizing solution" that are used in an embodiment of step b):

The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution

Id. at Col. 32:9-16.

46. A POSA would have understood that the use of these solutions with Compound 1 would not involve a direct reaction between Compound 1 and an acid to form Compound 2. Instead, the use of these solutions would result in multi-step and indirect reactions to form Compound 2. The figure below shows how these reactions would proceed:



For clarity, the structure containing three phenyl rings at the bottom of Compound 1 is the trityl group.

- 47. A POSA would have understood that the reaction described in the '322 Patent and shown above is a multi-step reaction. First, in the "acid treatment" portion of step b), Compound 1 reacts with the trifluoroacetic acid of the deblocking solution to remove the trityl group from Compound 1. However, the deblocking solution also contains 2,2,2-trifluoroethanol. A POSA would have understood that, as the "acid treatment" reaction progresses, the 2,2,2-trifluoroethanol would be capable of reacting with the trityl carbocation (structure shown in brackets above) that is formed upon addition of acid to compound 1 in order to indirectly drive the reaction forward according to Le Chatelier's principles of chemical equilibria. Thus, the "acid treatment" reaction involves an indirect, multistep reaction.
- 48. A POSA would also have understood that the result of the "acid treatment" reaction between Compound 1 and an acid would not be Compound 2. Instead, an intermediate compound

with a different chemical structure than Compound 2 (containing H<sub>2</sub><sup>+</sup> in place of the trityl group of Compound 1) would be formed. In order to form Compound 2, a POSA would have understood that this intermediate is reacted with the neutralizing solution during a neutralizing step. In view of these multiple steps, a POSA would understand that step b) does not require a "direct" reaction between Compound 1 and an acid to form Compound 2. In fact, the examples provided in the '322 Patent do not describe such a "direct" reaction. For the same reasons, a POSA would have understood that step e) does not require a Compound 3 to react directly with a deprotecting agent to form Compound 4.

- 49. A POSA would have understood that as long as the reaction described in Term 2 included both Compound 3 and a deprotecting agent—any number of additional (chemically reactive) reagents could be included in step e). For that reason, Sarepta's proposed inclusion of the word "directly" to describe the chemical reaction in Term 2 is not consistent with a POSA's understanding.
- 50. Second, I will address Sarepta's inclusion of the requirement that the Compound 3 recited in Term 2 be "of step d)."
- 51. Dr. Pentalute has argued that Sarepta's proposed construction is appropriate because "Step e) refers back to Compound 3 formed in step d) by expressly identifying it with 'said' Compound 3." Ex. 14 at ¶ 45. I disagree with Dr. Pentalute's interpretation of the claims.
- 52. As discussed above, Compound 3 is defined in claims 1 and 6 by its chemical structure—not by the final reaction used to form Compound 3:
  - d) repeating steps b) and c) until Compound 3 is complete:

- 53. While the result of step d) is Compound 3, a POSA would have understood that the claims expressly define Compound 3 by its chemical structure—not by the final reaction used to produce it.
- 54. Accordingly, a POSA would have understood that Term 2's use of "said Compound 3" refers to a Compound 3 having the same previously defined chemical structure and does not require Compound 3 to come from any specific source.
- 55. Indeed, a POSA would have understood that unrecited steps could be performed between steps d) and e) that could alter the result of step d). However, a POSA would have understood that as long as step e) used Compound 3 having the same chemical structure as was previously defined, it would meet the requirements of the claim.
- 56. For example, I understand that NS has provided the following hypothetical Method B to explain its position:

Step	Method A	Method B
Number	(Only Claimed Steps)	(Additional Unrecited Steps)

1	Step a) – Providing Compound 1	Step a) – Providing Compound 1	
2	Step b) – Forming Compound 2	Step b) – Forming Compound 2	
3	Step c) – Reacting Compound 2 with a monomer	Step c) – Reacting Compound 2 with a monomer	
4	Step d) – repeating steps b) and c) to form Compound 3	Step d) – repeating steps b) and c) to form Compound 3	
5		Reacting Compound 3 with a reagent to form an intermediate.	
6		Reacting the intermediate with a second reagent to re-form Compound 3.	
7	Step e) – Reacting Compound 3 with reagent to form Compound 4	Step e) – Reacting Compound 3 with reagent to form Compound 4	
8	Step f) – Reacting Compound 4 with acid to form the Oligomer or PMO	Step f) – Reacting Compound 4 with acid to form the Oligomer or PMO	

- 57. A POSA would be aware of several potential options that fit NS's hypothetical Method B.
- 58. In one example, a POSA could seek to avoid infringement under Sarepta's construction by performing a simple reaction to (i) remove the trityl group of Compound 3 in step 5 of Method B and (ii) replace it with a hydrogen:

- 59. Then in step 6 of Method B, the POSA could reverse the reaction and reform Compound 3 by re-adding the trityl group at the same position. Then the POSA could complete the method of claims 1 and 6 using Compound 3 of intermediate and unrecited step 6. Such a method—which would avoid infringement under Sarepta's proposed construction—demonstrates why Sarepta's proposal cannot be adopted.
- 60. Alternatively, after Compound 3 is created in step d), a POSA could modify the linker and/or solid carrier of Compound 3. For example, this is especially true if a POSA was interested in using a "safety catch" linker, which has advantages in allowing the final product to be more easily removed from the solid carrier with fewer side products. *See* Ex. 16, Ex. 17.
- 61. In step 5, a POSA could perform a reaction to chemically modify the "safety catch" linker. Such a reaction may result in unwanted modifications to the oligomer itself—for example protonation or alkylation of the purine bases. Then in step 6, the POSA would remove the unwanted modifications to the oligomer. Steps 5 and 6 would result in Compound 3 that meets

the chemical structure provided in the claims, but that is not "of step d)." The POSA could then complete the method of claims 1 and 6 using Compound 3 of the intermediate and unrecited step 6. Such a method—which would avoid infringement under Sarepta's proposed construction—further demonstrates why Sarepta's proposal cannot be adopted.

62. Since a POSA would have understood that Term 2 requires using Compound 3 that meets the chemical structure provided in the claims but does not require using the specific result of step d), Sarepta's proposed construction must be rejected.

VIII. Term 3 – "f) reacting Compound 4 with an acid to form said oligomer" or "f) reacting said Compound 4 with an acid to form said PMO"

63. The parties' proposed constructions for disputed Term 2 are shown below:

<u>Term</u>	NS's Position	Sarepta's Position
"f) reacting Compound 4 with an acid to form said oligomer" ('322 Patent Claim 1) or "f) reacting said Compound 4 with an acid to form said PMO" ('322 Patent Claim 6)	Plain and ordinary meaning – i.e., chemically reacting Compound 4 with an acid, in order to form the oligomer [or the PMO]	Plain and ordinary meaning, i.e., chemically reacting an acid directly with Compound 4 of step e), which results in the oligomer or the PMO.  Step f) must occur after step e).

64. In my opinion, a POSA would have understood Term 3 to mean "chemically reacting Compound 4 with an acid, in order to form the oligomer [or the PMO]" as is consistent with NS's proposed construction. I further note that replacing "in order to form the oligomer [or the PMO]" in NS's proposed construction with either "to form the oligomer [or the PMO]" or "which results in the oligomer [or the PMO]" would be consistent with how a POSA understood Term 3.

- 65. A POSA would have understood that Term 3 is not consistent with Sarepta's proposed construction for at least two reasons: (1) Sarepta's proposed construction adds the word "directly" to describe the chemical reaction, and (2) Sarepta's proposed construction requires that step f) use the specific Compound 3 that was the result "of step e)" and that step f) occur after step e).
- 66. First, I will address the addition of the word "directly" in Sarepta's proposed construction.
- 67. As described above with Term 2, nothing in the claim language excludes the use of additional, unlisted reagents as part of the reaction of step f). Term 3 specifically uses the word "reacting," which a POSA would understand is not limited to only "direct reactions" between the two listed reagents. Indeed, a POSA would understand that certain reactions necessarily involve more than two reagents.
- 68. For example, step f) contains nearly identical language as step b), and both steps serve the purpose of removing a trityl group from the compound:
  - b) reacting said Compound 1 with an acid to form Compound 2;
  - f) reacting Compound 4 with an acid to form said oligomer.

#### '322 Patent at cl. 1.

69. As I discussed in detail above, step b) can be a multi-step reaction rather than a "direct" reaction between Compound 1 and an acid—and the example of step b) in the '322 Patent describes such a multi-step reaction. Step f) can be a multi-step reaction for the same reasons. Like with Compound 1 of step b), when Compound 4 of step f) is reacted with an acid, the result may be an intermediate compound containing H<sub>2</sub><sup>+</sup> in place of the trityl group of Compound 4. A

POSA would have understood that the intermediate compound would need to be neutralized with a base to obtain the claimed oligomer or PMO. Indeed, the specification of the '322 Patent explains that such neutralization may be required to obtain the final oligomer or PMO:

PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C<sub>8</sub> to C<sub>18</sub>, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., e.g., WO 1991/09033).

'322 Patent at Col. 23:57-67.

- 70. Thus, a POSA would have understood that step f) could include multi-step or indirect reactions, and was not limited to only "direct" reactions between Compound 4 and an acid.
- 71. A POSA would have understood that as long as the reaction described in Term 3 included both Compound 4 and an acid—any number of additional (chemically reactive) reagents could be included. For that reason, Sarepta's proposed inclusion of the word "directly" to describe the chemical reaction in Term 3 is not consistent with a POSA's understanding.
- 72. Second, I will address Sarepta's inclusion of the requirement that the Compound 4 recited in Term 3 be "of step e)."
- 73. Dr. Pentalute has argued that Sarepta's proposed construction is appropriate because "it is logical that step e) is performed before step f) to obtain 'said Compound 4' used in step." Ex. 14 at ¶ 71. I disagree with Dr. Pentalute's interpretation of the claims.
- 74. As discussed above, Compound 4 is defined in claims 1 and 6 by its chemical structure—not by the final reaction used to form Compound 4:
  - e) reacting said Compound 3 with a deprotecting agent to form Compound 4:

[Compound 4]

- 75. While the result of step e) is Compound 4, a POSA would have understood that the claims expressly define Compound 4 by its chemical structure—not by the final reaction used to produce it.
- 76. Accordingly, a POSA would have understood that Term 3's use of "said Compound 4" (or just "Compound 4" in the case of claim 1) refers to a Compound 4 having the same previously defined structure and does not require Compound 4 to come from any specific final reaction.
- 77. Indeed, a POSA would have understood that unrecited steps could be performed between steps e) and f) that could alter the result of step e). However, a POSA would have

understood that as long as step f) used Compound 4 having the same chemical structure as was previously defined, it would meet the requirements of the claim.

- 78. For example, a POSA could seek to avoid infringement under Sarepta's proposed construction by performing a transient silylation with TMS. *See* Ex. 18. This reaction will modify the 5' alcohol group and some of the nucleobases of Compound 4 making it more hydrophobic. This could be useful for purification because a POSA could then use regular, silica-gel chromatography to purify the resulting product. After chromatography, the POSA would treat the purified product with dilute ammonia to re-form Compound 4—this time in a more purified state. I routinely perform such reactions in my lab with small molecules—and given the neutral backbone of the PMOs of claims 1 and 6, the same series of reactions should work with Compound 4.
- 79. After obtaining the purified Compound 4, the POSA could complete the method of claims 1 and 6 using Compound 4 of this intermediate and unrecited step. Such a method—which would avoid infringement under Sarepta's proposed construction—further demonstrates why Sarepta's proposal cannot be adopted.
- 80. Moreover, a POSA would have understood that steps e) and f) could be performed in any order. These polishing or finishing steps are dependent on the purification methods available to the POSA. A POSA would have understood that a variety of different purification schemes could be used, and that the order of steps e) and f) is not relevant to the method.
- 81. Since a POSA would have understood that Term 3 requires using Compound 4 that meets the chemical structure provided in the claims but does not require using the specific result of step e), Sarepta's proposed construction must be rejected.

#### IX. Conclusion

82. I declare under penalty of perjury under the laws of the United States that the foregoing is true and correct. Executed at Mont-Royal, Quebec this 27th day of February, 2023.

Nathan W. Luedtke, Ph.D

# EXHIBIT 16

Shane T. Flickinger, $^{\uparrow,\#}$  Madhusudan Patel, $^{\uparrow,\S,\#}$  Brock F. Binkowski, $^{\ddagger}$  Aaron M. Lowe, $^{\perp,\S}$  Mo-Huang Li, $^{\P,\S}$  Changhan Kim, $^{\P,\S}$  Franco Cerrina, $^{\P,\S}$  and Peter J. Belshaw\*, $^{\uparrow,\ddagger}$ 

The Department of Chemistry, The Department of Biochemistry, The Department of Computer and Electrical Engineering, Materials Science Program, and Center for Nanotechnology, University of Wisconsin—Madison, Madison, Wisconsin 53706

belshaw@chem.wisc.edu

Received March 15, 2006

Downloaded via MCGILL UNIV on February 15, 2023 at 17:19:31 (UTC). See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

#### **ABSTRACT**

We report the development of a safety-catch photolabile linker that allows the light-directed synthesis and spatially selective photorelease of oligonucleotides from microarrays. The linker remains stable to light during DNA synthesis, and is activated for photorelease after acidic hydrolysis. We demonstrate that the photoreleased oligonucleotides can be amplified by PCR to produce double stranded DNA. The advantages offered by this linker could aid the development of an automated gene synthesis platform.

The in situ, light-directed synthesis of oligonucleotides in microarrays, first introduced by Fodor and co-workers at Affymetrix, has become a powerful diagnostic tool. In this approach, oligonucleotide microarrays are synthesized through iterative deprotection and coupling cycles, using nucleoside phosphoramidites that can be deprotected directly or indirectly with light. More recently, the photodeprotections have been mediated by a maskless array synthesizer (MAS). The MAS uses light reflected from a digital micromirror device, enabling the production of oligonucleotide arrays with

up to 786 432 features. This approach allows for the rapid and flexible synthesis of an oligonucleotide microarray with any desired set of sequences, inviting the prospect of utilizing oligonucleotides synthesized in microarrays as templates for gene assembly (Figure 1, Supporting Information). Indeed, several methods for gene assembly from microarrays have already been reported.<sup>4</sup>

A method to selectively release oligonucleotides in a controlled, spatial manner from subregions of a microarray

<sup>†</sup> Department of Chemistry.

<sup>&</sup>lt;sup>‡</sup> Department of Biochemistry.

<sup>§</sup> Center for Nanotechnology.

 $<sup>^{\</sup>perp}$  Materials Science Program.

<sup>&</sup>lt;sup>¶</sup> Department of Computer and Electrical Engineering.

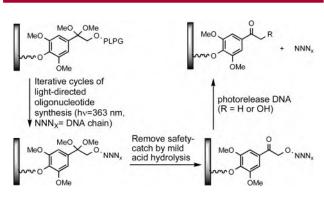
<sup>#</sup> These authors contributed equally to this work.

<sup>(1)</sup> Pease, A. C.; Solas, D.; Sullivan, E. J.; Cronin, M. T.; Holmes, C.

<sup>P.; Fodor, S. P.</sup> *Proc. Natl. Acad. Sci. U.S.A.* 1994, 91, 5022-5026.
(2) (a) McGall, G.; Labadie, J.; Brock, P.; Wallraff, G.; Nguyen, T.; Hinsberg, W. *Proc. Natl. Acad. Sci. U.S.A.* 1996, 93, 13555-13560. (b) Gao, X. L.; Yu, P. L.; LeProust, E.; Sonigo, L.; Pellois, J. P.; Zhang, H. *J. Am. Chem. Soc.* 1998, 120, 12698-12699. (c) Giegrich, H.; Eisele-Buhler, S.; Hermann, C.; Kvasyuk, E.; Charubala, R.; Pfleiderer, W. *Nucleosides Nucleotides* 1998, 17, 1987-1996.

<sup>(3) (</sup>a) Singh-Gasson, S.; Green, R. D.; Yue, Y. J.; Nelson, C.; Blattner, F.; Sussman, M. R.; Cerrina, F. Nat. Biotechnol. 1999, 17, 974–978. (b) LeProust, E.; Pellois, J. P.; Yu, P. L.; Zhang, H.; Gao, X. L.; Srivannavit, O.; Gulari, E.; Zhou, X. C. J. Comb. Chem. 2000, 2, 349–354. (c) Luebke, K. J.; Balog, R. P.; Mittelman, D.; Garner, H. R. In Microfabricated Sensors, Application of Optical Technology for DNA Analysis; Kordal, R., Usmani, A., Law, W. T., Eds.; American Chemical Society: Washington, DC, 2002; Vol. 815, pp 87–106.

<sup>(4) (</sup>a) Richmond, K. E.; Li, M.; Rodesch, M. J.; Patel, M.; Lowe, A. M.; Kim, C.; Chu, L. L.; Venkataramaian, N.; Flickinger, S. T.; Kaysen, J.; Belshaw, P. J.; Sussman, M. R.; Cerrina, F. *Nucleic Acids Res.* **2004**, *32*, 5011–5018. (b) Tian, J.; Gong, H.; Sheng, N.; Zhou, X.; Gulari, E.; Gao, X.; Church, G. *Nature* **2004**, *432*, 1050–1054. (c) Zhou, X.; Cai, S.; Hong, A.; You, Q.; Yu, P.; Sheng, N.; Srivannavit, O.; Muranjan, S.; Rouillard, J. M.; Xia, Y.; Zhang, X.; Xiang, Q.; Ganesh, R.; Zhu, Q.; Matejko, A.; Gulari, E.; Gao, X. *Nucleic Acids Res.* **2004**, *32*, 5409–5417.



**Figure 1.** Use of a safety-catch photolabile linker for light-directed synthesis and release of oligonucleotides.

could aid the development of a robust gene assembly platform. Chemical methods of elution, such as the basic hydrolytic cleavage of linkers,<sup>5</sup> have the disadvantage of releasing oligonucleotides from all exposed areas of the array surface. Selective release of oligonucleotides from the array surface could be accomplished through a microfluidic approach, such that a cleavage reagent is delivered only to specific regions of the array. Here we report a safety-catch photolabile linker (SC-PLL) that allows for both light-directed synthesis and spatially selective photorelease of oligonucleotides in microarrays.

A review of photolabile protecting groups (PLPGs) and photolabile linkers (PLLs)<sup>6</sup> presented two potential strategies for linker design: (1) wavelength selective photolysis or (2) latent photocleavable linkers. The independent, orthogonal deprotection of PLPGs has been achieved through the use of compounds that respond selectively to light of differing wavelengths.<sup>7</sup> However, the reported selectivity is not absolute and is limited by the broad absorption spectra of these photolabile protecting groups. As the synthesis of an oligonucleotide microarray requires many irradiation cycles prior to release, wavelength selective release was not deemed practical. SC-PLL's based on the benzoin PLPG have previously been developed to reduce light-sensitivity and improve the chemical stability of PLPGs used in syntheses.<sup>8</sup>

A SC-PLL for oligonucleotide synthesis and release must be compatible with the conditions of DNA synthesis, and activation/photolysis conditions must be compatible with DNA. We chose to develop our safety-catch based on the phenacyl PLPG<sup>9</sup> since masking the carbonyl as a dimethyl ketal renders this group photoinert during oligonucleotide

synthesis. Postsynthesis activation of the safety-catch by mild acidic hydrolysis enables selective photorelease (Figure 1).

Synthesis of the SC-PLL (Scheme 1) began with 3',5'-dimethoxy-4'-hydroxyacetophenone 1 and ethylene glycol. Ethylene glycol was monoprotected with TBS-Cl, and then coupled to 1 via Mitsunobu reaction to yield 2 (87%). Treatment of 2 with PhI(OAc)<sub>2</sub> in basic MeOH gave 3 (84%). The free alcohol was protected with NPPoc-Cl<sup>11</sup> and deprotected with TBAF to give 4 (89%). Finally, treatment with 2-cyanoethyl diisopropylchlorophosphoramidite gave phosphitlylated linker 5 (86%), ready for attachment to functionalized surfaces. 12

To investigate the conditions for activation and photorelease of SC-PLL 5, monohydroxysilane derivatized glass slides<sup>3a</sup> were prepared and extended with two NPPochexaethylene glycol-phosphoramidite spacers (analogous to previously reported MeNPoc-hexaethyleneglycol-phosphoramidites), 11c followed by coupling of the SC-PLL 5. Intermediate phosphites were oxidized to the phosphotriesters with I<sub>2</sub>/H<sub>2</sub>O/pyridine solution. A series of square features containing T20-oligomers were synthesized via standard protocols,13 using iterative cycles of photodeprotection and coupling/oxidation of NPPoc-protected nucleoside phosphoramidites (Proligo), followed by deprotection with a 1:1 solution of ethylenediamine and ethanol. Small squares inside the larger square features were irradiated for varying times either before or after hydrolysis of the dimethyl ketal with acid. For acid activation of the linker, 3% trichloroacetic acid (w/v) in 95:5 acetone/MeOH was used to minimize nonspecific stripping from the glass slides and potential depurination of oligonucleotides, while allowing rapid activation of the linker. Thioxanthone or 1-chloro-4-propoxy-9*H*-thioxanthone (CPT) was used to enable the efficient, long wavelength (>360 nm) photolysis of the phenacyl group. The exact mechanism of thioxanthone mediated photorelease remains undetermined; however, it may function as a photoreductant or as a triplet sensitizer.14 The slide was visualized by hybridization to a complementary Cy3-labeled A<sub>29</sub> probe (Figure 2a). Selective and spatially controlled photorelease of oligonucleotides was revealed by loss of fluorescence in small inner squares only after acid activation of the safetycatch. No photorelease of the oligomer was observed prior to acid activation.

2358 Org. Lett., Vol. 8, No. 11, 2006

<sup>(5)</sup> Beaucage, S. L.; Iyer, R. P. Tetrahedron 1992, 48, 2223-2311.

<sup>(6) (</sup>a) Givens, R. S.; Conrad, P. G., II; Yousef, A. L.; Lee, J.-I. In *CRC Handbook of Organic Photochemistry and Photobiology*, 2nd ed.; Horspool, W. M., Lenci, F., Eds.; CRC Press: Boca Raton, FL, 2004, Chapter 69. (b) Bochet, C. G. *J. Chem. Soc.*, *Perkin Trans. 1* 2002, 125–142. (c) Falvey, D. E.; Sundararajan, C. *Photochem. Photobiol.* 2004, *3*, 831–838.

<sup>(7)</sup> Bochet, C. G. Synlett 2004, 2268-2274.

<sup>(8) (</sup>a) Rock, R. S.; Chan, S. I. *J. Org. Chem.* **1996**, *61*, 1526–1529. (b) Routledge, A.; Abell, C.; Balasubramanian, S. *Tetrahedron Lett.* **1997**, *38*, 1227–1230. (c) Lee, H. B.; Balasubramanian, S. *J. Org. Chem.* **1999**, *64*, 3454–3460. (d) Cano, M.; Ladlow, M.; Balasubramanian, S. *J. Comb. Chem.* **2002**, *4*, 44–48. (e) Cano, M.; Ladlow, M.; Balasubramanian, S. *J. Org. Chem.* **2002**, *67*, 129–135.

<sup>(9) (</sup>a) Sheehan, J. C.; Umezawa, K. *J. Org. Chem.* **1973**, *38*, 3771–3774. (b) Conrad, P. G.; Givens, R. S.; Weber, J. F. W.; Kandler, K. *Org. Lett.* **2000**, *2*, 1545–1547.

<sup>(10)</sup> Moriarty, R. M.; Hou, K. *Tetrahedron Lett.* **1984**, 691–694.

<sup>(11) (</sup>a) Hasan, A.; Stengele, K. P.; Giegrich, H.; Cornwell, P.; Isham, K. R.; Sachleben, R. A.; Pfleiderer, W.; Foote, R. S. *Tetrahedron* **1997**, *53*, 4247–4264. (b) Pirrung, M. C.; Wang, L. X.; Montague-Smith, M. P. *Org. Lett.* **2001**, *3*, 1105–1108. (c) McGall, G. H.; Barone, A. D.; Diggelmann, M.; Fodor, S. P. A.; Gentalen, E.; Ngo, N. *J. Am. Chem. Soc.* **1997**, *119*, 5081–5090.

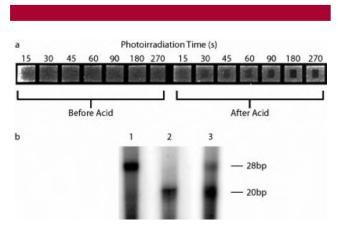
<sup>(12)</sup> Atkinson, T.; Smith, M. In *Oligonucleotide Synthesis: a Practical Approach*; Gait, M. J., Ed.; IRL Press: Oxford, UK, 1984; pp 35–83.

<sup>(13)</sup> Nuwaysir, E. F.; Huang, W.; Albert, T. J.; Singh, J.; Nuwaysir, K.; Pitas, A.; Richmond, T.; Gorski, T.; Berg, J. P.; Ballin, J.; McCormick, M.; Norton, J.; Pollock, T.; Sumwalt, T.; Butcher, L.; Porter, D.; Molla, M.; Hall, C.; Blattner, F.; Sussman, M. R.; Wallace, R. L.; Cerrina, F.; Green, R. D. *Genome Res.* **2002**, *12*, 1749–1755.

<sup>(14)</sup> Woll, D.; Walbert, S.; Stengele, K. P.; Albert, T. J.; Richmond, T.; Norton, J.; Singer, M.; Green, R. D.; Pfleiderer, W.; Steiner, U. E. *Helv. Chim. Acta* **2004**, 87, 28–45.

#### **Scheme 1.** Synthesis of the SC-PLL

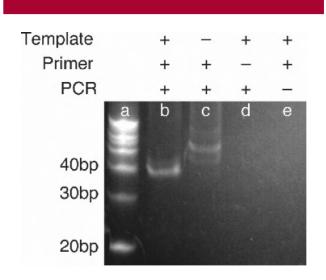
To more directly demonstrate spatial photorelease and assess the integrity of the released oligonucleotides we prepared an array with four features containing  $T_{20}$  and  $T_{28}$ oligomers (Figure 4, Supporting Information). After acid activation, approximately half of each oligonucleotide region was photoreleased and collected separately. The T<sub>28</sub> oligomer was photoreleased first followed by release of the T<sub>20</sub> oligomer. The remaining oligonucleotides on the slide were chemically released with concentrated ammonium hydroxide. 15 The oligonucleotides were radiolabeled with 32P ATP and analyzed by polyacrylamide gel electrophoresis. The gel shows the spatially selective release of the T<sub>28</sub> (Figure 2b, lane 1) and T<sub>20</sub> oligomers (Figure 2b, lane 2). The chemical release (Figure 2b, lane 3) shows the expected indiscriminate release of both the T<sub>28</sub> and T<sub>20</sub> oligomers. The gel profiles also show that the major species released from the slide are the desired  $T_{28}$  and  $T_{20}$  oligomers. These experiments



**Figure 2.** (a) Fluorescence scan of a series of  $T_{20}$  oligomer features (large squares, 640  $\mu$ m<sup>2</sup>) revealing selective, spatial photorelease of the  $T_{20}$  oligomers (small squares, 280  $\mu$ m<sup>2</sup>) after acid treatment as visualized by hybridization to a complementary Cy3-labeled  $A_{29}$  probe. (b) Denaturing electrophoresis gel profile of 5'-<sup>32</sup>P-labeled  $T_{20}$  and  $T_{28}$  oligomers synthesized and photoreleased from the DNA microarray showing selective synthesis and photorelease of oligonucleotides. The SC-PLL was activated with 3% trichloroacetic acid (w/v) in 95:5 acetone/MeOH (20 min) and photoreleased in the presence of 0.01% CPT (270 s). Lane 1, photoreleased  $T_{28}$ ; lane 2, photoreleased  $T_{20}$ ; and lane 3, chemical release of  $T_{20}$  and  $T_{28}$  oligomers from the microarray (concentrated NH<sub>4</sub>OH, 4 h, rt).

demonstrate that the SC-PLL enables the spatial synthesis and spatial release of oligonucleotides in microarrays.

To investigate whether the photoreleased oligonucleotides are functional as templates for amplification, we synthesized and photoreleased a mixed base 40-mer that served as a template for PCR amplification. Figure 3 shows the PCR



**Figure 3.** Acrylamide gel profile of a mixed base 40mer photoreleased from a microarray and amplified by PCR. Lane a, oligonucleotide markers (Amersham Biosciences); lane b, photoreleased 40mer amplified by PCR; lane c, water control (no template); lane d, template (no primer); and lane e, photoreleased 40mer (no PCR). Sequence of 40mer: 5'-TGC-CGG-AGT-CAG-CGT-AGG-ATA-TCT-CGT-GGC-GAC-TCT-GAC-T-3'.

products analyzed by acrylamide gel electrophoresis. A major band of 40 bp in length appears only in the PCR reaction containing primers and the photoreleased template. The successful demonstration of PCR amplification of the photoreleased oligonucleotides should enable studies toward multiplexed gene assembly from a single microarray. <sup>16</sup>

Org. Lett., Vol. 8, No. 11, 2006

<sup>(15)</sup> LeProust, E.; Zhang, H.; Yu, P. L.; Zhou, X. C.; Gao, X. L. *Nucleic Acids Res.* **2001**, *29*, 2171–2180.

## Case 1:21-cv-01015-JLH Document 174-2 Filed 03/20/23 Page 272 of 318 PageID #: 7373

The modular design of our SC-PLL (Figure 2, Supporting Information) should enable the linker to be tuned for particular applications. The acid sensitivity of the safety-catch can be tuned by varying the nature of the ketal; NPPoc could easily be replaced by another PLPG; and the method of surface attachment can be modified. Although we currently employ thioxanthones to enable long-wavelength photolysis (>360 nm), sensitizing groups have also been incorporated directly into PLPGs.<sup>17</sup> In addition, our SC-PLL is not limited to light-directed oligonucleotide synthesis. It should be possible to mask the PLL with a safety-catch that can be removed via basic, oxidative, or reductive methods, <sup>18</sup> extending its utility to other microarray synthesis formats.<sup>4b,c,19</sup>

There are several potential advantages of using SC-PLL **5** for gene assembly in a microarray format. First, the SC-PLL gives, in theory, complete flexibility with regard to which sequences are released from the microarray, possibly enabling multiplexed PCR assembly reactions on subsets of photoreleased oligonucleotides from a single microarray. Second, SC-photorelease could potentially improve the fidelity of oligonucleotides eluted from the array surface by limiting the release of error containing sequences formed at the boundaries of features, due to partial deprotections and stray light.<sup>20</sup> This could be accomplished by selectively releasing the internal region of a photopatterned feature without releasing oligonucleotides at the edges of the feature (Figure 3, Supporting Information). Similarly, with an

incomplete photorelease (e.g., photoreleasing to 50% completion), the higher quality oligonucleotides should be selectively released from the regions with highest light intensity within each pixel. Finally, using a SC-PLL to release oligonucleotides from subregions of the array should significantly reduce the number of unique primer sets needed for PCR based amplification. In addition, for small assemblies, a SC-PLL could eliminate the need for primer sets altogether.

In summary, our experiments collectively demonstrate that SC-PLL **5** enables the spatially arrayed synthesis and spatially controlled photorelease of oligonucleotides in microarrays, and that the oligonucleotides released are functional as templates for PCR amplification. Additionally, using a SC-PLL may improve the fidelity of eluted oligonucleotides, and reduce the number of primer sets needed for subsequent assembly reactions. These unique features will aid the development of an automated gene synthesis platform. The assembly of spatially released oligonucleotides from the SC-PLL into short double stranded DNA fragments as well as the design and synthesis of a SC-PLL that functions at long wavelengths (>360 nm) without the use of a sensitizer are areas of ongoing research.

Acknowledgment. We gratefully acknowledge DARPA (DAAD 19-02-2-0026), the NIH (NIHGR 1R01HG003275), and the W. M. Keck Foundation for financial support of this work and NSF (CHE-9208463) and NIH (RR08389) for support of NMR facilities. We thank Dr. K. Richmond (Center for Nanotechnology, UW-Madison) for advice and PCR primers, and the L. M. Smith (Department of Chemistry, UW-Madison) group for use of their fluorescence scanner.

**Supporting Information Available:** Figures, synthesis, and spectra of SC-PLL **5**, surface chemistry, and PCR. This material is available free of charge via the Internet at http://pubs.acs.org.

OL060644X

2360 Org. Lett., Vol. 8, No. 11, 2006

<sup>(16)</sup> Kim, C.; Richmond, K. E.; Rodesch, M. J.; Binkowski, B.; Chu, L. L.; Li, M.-H.; Heinrich, K.; Blair, S.; Belshaw, P. J.; Sussman, M. R.; Cerrina, F. *Microelectron. Eng.* In press.

<sup>(17)</sup> Lee, K.; Falvey, D. E. *J. Am. Chem. Soc.* **2000**, *122*, 9361–9366. (18) (a) Shaginian, A.; Patel, M.; Li, M.-H.; Flickinger S. T.; Kim, C.; Cerrina, F.; Belshaw P. J. *J. Am. Chem. Soc.* **2004**, *126*, 16704–16705. (b) Stowell, M. H. B.; Wang, G.; Day, M. W.; Chan, S. I. *J. Am. Chem. Soc.* **1998**, *120*, 1657–1664.

<sup>(19)</sup> Cleary, M. A.; Kilian, K.; Wang, Y.; Bradshaw, J.; Cavet, G.; Ge, W.; Kulkarni, A.; Paddison, P. J.; Chang, K.; Sheth, N.; Leproust, E.; Coffey, E. M.; Burchard, J.; McCombie, W. R.; Linsley, P.; Hannon, G. J. *Nature Methods* **2004**, *1*, 241–248.

<sup>(20)</sup> Garland, P. B.; Serafinowski, P. J. Nucleic Acids Res. 2002, 30, e99.

## EXHIBIT 17

Name: Franziska Weichelt

**Topic: Safety-CAtch Linker (SCAL)** 

**Related Topics:** Linker types (monofunctional linker, multidirectional linker, cleavage linker)

#### **Definition:**

The term "safety catch principle" was introduced by Kenner in 1971 for peptide chemistry to describe a strategy that allows a linker to remain stable until it is activated for cleavage by a chemical modification.

Currently, the term safety-catch is applied to a linker that is cleaved by performing two different reactions instead of the normal single step, that means that the linker is unreactive to the conditions of the library synthesis but may be activated by a simple chemical transformation to permit cleavage of the library from the solid phase to take place. Safety-catch linkers are used especially for solid-phase-syntheses, which are nowadays very important and can be used for nearly every known reaction (syntheses of alcohols, phenols, guanidines, aldehydes, carboxylic acids, amides, esters, ...).

#### Advantages of safety-catch linkers:

- greater control over the timing of compound release
- stable to both strongly acidic and basic conditions
- the linker group is often reduced to a single atom
- sufficient stability of the linker-substrate-bond impedes hydrolysis or similar side reactions
- mild chemical conditions allowing unscathed liberation of the precious products

Developments regarding the linker, coupling conditions, activation methods, cleavage conditions and possible substrates established this linker and its modifications in modern chemistry and will without doubt be present in the future.

#### Mechanism [1]:



#### Examples:

- sulfone-linker for solid-phase synthesis of the 2-Aminobenzoxazole Library
- Kenner's safety-catch linker for N-Acyl-N-alkyl-sulfonamide anchors and for synthesis of primary amides, hydrazones and carboxylic acids [4]

• reverse Kenner safety-catch linker for Suzuki coupling and thiazolidinone formation [4]

• sulfide safety-catch linker [3]

- selenium safety-catch linkers for annulations, Knoevenagel condensation, Pd-catalyzed cross couplings, reductive aminations, Mitsonobu inversions, glycosidations, organometallic additions
- safety-catch linker for Hofmann-elimination [3]

#### **Publications:**

- [1] Combinatorial Chemistry
  - N. K. Terret, 1998, Oxford Chemistry Masters
- [2] Combinatorial Chemistry A practical approach W. Bannwarth, E. Felder, 2000, Wiley-VCH

#### Web-Links:

- [3] http://phoenix.tuwien.ac.at/combichem/Combi\_2004\_5\_linkers2.pdf
- [4] http://www.scs.uiuc.edu/chem/gradprogram/chem435/spring03/Elmer.pdf
- [5] http://www.combichemistry.com/glossary\_s.html
- [6] http://www.mdpi.net/ecsoc/ecsoc-5/Papers/e0027/e0027.htm
- [7] http://www.5z.com/moldiv/volume5/abstract25-34.pdf

# EXHIBIT 18

#### Transient Protection: Efficient One-Flask Syntheses of Protected Deoxynucleosides<sup>1</sup>

G. S. Ti, B. L. Gaffney, and R. A. Jones\*,

Contribution from the Department of Chemistry, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854. Received March 3, 1981

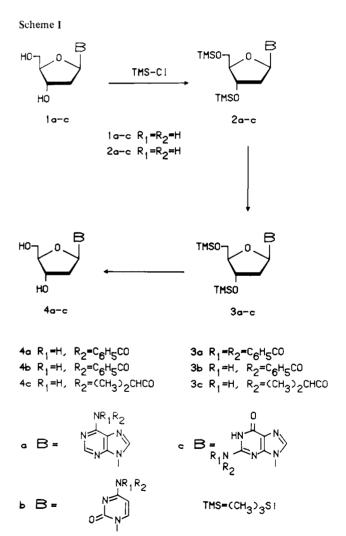
Abstract: Application of the concept of transient protection to the synthesis of protected deoxynucleosides is described. The deoxynucleosides are first treated with trimethylchlorosilane in pyridine for protection of the hydroxyl groups, and then immediately reacted with an acylating agent—benzoyl chloride for 1a and 1b and isobutyric anhydride for 1c—to effect N-acylation. Hydrolysis of the trimethylsilyl groups takes a few hours in aqueous pyridine or a few minutes with dilute ammonia. The ammonia also effects selective hydrolysis of the initially formed N,N-dibenzoyldeoxyadenosine derivative (3a) to the desired N-benzoyldeoxyadenosine (4a). This one-flask procedure gives crystalline N-acyl deoxynucleosides 4a and 4b in 95% yield and 4c in 75% yield, in only a few hours. The 5'-O-dimethoxytrityl deoxynucleosides 8a and 8b are also obtained in a one-flask procedure by initial reaction of the deoxynucleosides with 4,4'-dimethoxytrityl chloride, followed by treatment with trimethylchlorosilane and then benzoyl chloride. Although with deoxycytidine some of the 4-N,5'-O-bis(dimethoxytrityl) derivative (5c) is formed, benzoyl chloride effects conversion to the 4-N-benzoyl derivative (7b). After simple purification by flash chromatography 8a and 8b are each obtained in 80-90% overall yield from 1a or 1b.

Synthetic oligonucleotides have proved to be invaluable tools of modern molecular biology.<sup>2</sup> Their uses include enzymatic construction of genes,<sup>3-7</sup> most recently an interferon gene,<sup>8</sup> physical studies of the stability and conformational behavior of nucleic acids, 9-14 and investigation of drug-nucleic acid 15 and protein-nucleic acid interactions. 16 Recent advances in chemical synthesis, 17-19 especially polymer-supported methods, 20-22 have markedly reduced the enormous amount of time and effort formerly required for oligonucleotide synthesis. 23,24 The effort devoted to protection of the monomers has become a significant aspect of a total synthesis. In fact, it may now be possible to synthesize an oligomer, by solid phase, in less time than it takes to protect the deoxynucleosides used in the synthesis.

The protecting group methodology and procedures still employed, in either solution or solid-phase synthesis, by either the triester or phosphite methods, are those originally developed by Khorana and co-workers for their diester method. 25,26 In this scheme the nucleosides are peracylated and the O-acyl groups then selectively cleaved with controlled hydroxide treatment to give the N-acyl derivatives 4a-c. The 5'-hydroxyl is then protected with either an acid labile trityl derivative, usually the 4,4'-dimethoxytrityl group, or with the 2-(dibromomethyl)benzoyl group introduced recently by Reese.<sup>17</sup> Although this is a proven, reliable procedure, it is multi-step. As a result it is time consuming and gives only moderate overall yields. Attempts at selective Nacylation with use of benzoic anhydride, 27 2-(chloromethyl)-4nitrophenyl benzoate,28 O-ethyl S-benzoyl dithiocarbonate,29 p-nitrophenyl benzoate/1-hydroxybenzotriazole,30 or pentafluorophenyl benzoate<sup>31</sup> have been successful only with cytosine nucleosides. With other nucleosides concomitant O-acylation invariably accompanies N-acylation.

Several ongoing projects in our laboratory require that we incorporate into oligonucleotides specifically deuterated or otherwise modified nucleosides which we have synthesized. We therefore sought a method for protecting nucleosides that would be both faster and more nucleoside efficient than the Khorana procedure. An obvious way to achieve overall selective N-acylation would be to protect the hydroxyl groups prior to the acylation reaction. This approach, however, would offer no improvement over the classic procedure unless the hydroxyl protecting groups could be much more easily removed than O-acyl groups.

We now wish to report application to this problem of the concept of transient protection. In this case such protection requires a protecting group which can be introduced quantitatively and is selective for the hydroxyl groups. Further, while it must be stable



during N-acylation in anhydrous pyridine it should be hydrolyzed in aqueous pyridine. High-yield synthesis of the N-acyl nucleosides

<sup>&</sup>lt;sup>†</sup>Address correspondence to this author at the Department of Chemistry, Douglass College, Rutgers University, New Brunswick, NJ 08903

<sup>(1)</sup> This work has been reported briefly, see "Abstracts of Papers", 181st National Meeting of the American Chemical Society, Atlanta, Georgia, April 1981; American Chemical Society, Washington, D.C., 1981; CARB 38.
(2) H. G. Khorana, H. Büchi, H. Chosh, N. Gupta, T. M. Jacob, H.

Kössel, R. Morgan, S. A. Narang, F. Ohtsuka, and R. D. Wells, Cold Spring Harbor Symp. Quant. Biol., 31, 39 (1966).

4a-c in one-flask reactions, using trimethylchlorosilane for transient hydroxyl protection, is shown in Scheme I.

Formation of the putative 3',5'-O-bis(trimethylsilyl) derivatives 2a-c occurs in minutes (TLC) upon addition of trimethylchlorosilane<sup>32</sup> to a suspension of the deoxynucleoside (1a-c) in dry pyridine. These necessarily labile compounds have not been isolated and the structures shown must therefore be presumed on the basis of subsequent reactions. Implicit in the criteria for transient protection is that the reagent, in this case trimethylchlorosilane, must not interfere with subsequent reactions. In fact, N-acylation is unaffected by the presence of even a substantial excess of trimethylchlorosilane. We have used benzoyl chloride for protection of deoxyadenosine and deoxycytidine and isobutyric anhydride for protection of deoxyguanosine. In each case the acylation is complete within 2 h. The reaction mixture is then cooled to 0 °C and quenched with water. Under these conditions hydrolysis of the trimethylsilyl groups requires about 4 h.

Reaction of adenine nucleosides with benzoyl chloride is known<sup>26</sup> to give N,N-dibenzoyl derivatives, in this case 3a, and we have isolated N,N-dibenzoyl-2'-deoxyadenosine from this reaction. However, selective hydrolysis of one of the benzoyl groups is achieved by brief treatment with 2 M aqueous ammonia, with no trace of complete N-deprotection. Ammonia also effects instant removal of the trimethylsilyl groups and for this reason is employed

(3) K. L. Agarwal, H. Büchi, M. H. Caruthers, N. Gupta, H. G. Khorana, K. Kleppe, A. Kumar, E. Ohtsuka, U. L. Rajbhandary, J. H. van de Sande, V. Sgaramella, H. Weber, and T. Yamada, Nature (London), 227, 27 (1970).

(4) K. Itakura, T. Hirose, R. Crea, A. D. Riggs, H. L. Heyneker, F. Bolivar, and H. W. Boyer, *Science*, 198, 1056 (1977).

(5) H. G. Khorana, Science, 203, 614 (1979). (6) D. V. Goeddel, H. L. Heyneker, T. Hozumi, R. Arentzen, K. Itakura, D. G. Yansura, M. J. Ross, G. Miozzari, R. Crea, and P. Seeburg, Nature (London), 281, 544 (1979).

(7) D. V. Goeddel, D. G. Kleid, F. Bolivar, H. L. Heyneker, D. Yansura, R. Crea, T. Hirose, A. Kraszewski, K. Itakura, and A. Riggs, Proc. Natl. Acad. Sci. U.S.A., 76, 106 (1979).

(8) M. D. Edge, A. R. Greene, G. R. Heathcliffe, P. A. Meacock, W. Schuch, D. B. Scanlon, T. C. Atkinson, C. R. Newton, and A. F. Markham, Nature (London), 292, 756 (1981).

(9) D. J. Patel and A. E. Tonelli, Biochemistry, 14, 3990 (1975).

(10) A. H.-J. Wang, G. J. Quigley, F. J. Kolpak, J. L. Crawford, J. H. van Boom, G. vd Marel, and A. Rich, Nature (London), 282, 680 (1979)

(11) F. H. Martin and I. Tinoco, Jr., Nucleic Acids Res., 8, 2295 (1980). (12) L. A. Marky, L. Canuel, R. A. Jones, and K. J. Breslauer, Biophys.

Chem., 13, 141 (1981)

(13) H. R. Drew, R. M. Wing, T. Takano, C. Broka, S. Tanaka, K. Itakura, and R. E. Dickerson, Proc. Natl. Acad. Sci. U.S.A., 78, 2179 (1981). (14) L. A. Marky, K. Blumenfeld, and K. J. Breslauer, Biopolymers, in

press. (15) D. J. Patel, S. A. Kozlowski, J. A. Rice, L. A. Marky, K. J. Breslauer, C. Broka, and K. Itakura, in "Topics in Molecular and Structural Biology II", W. Fuller and S. Neidle, Eds., MacMillan, New York, 1982.

(16) M. H. Caruthers, Acc. Chem. Res., 13, 155 (1980) (17) J. B. Chattopadhyaya and C. B. Reese, Nucleic Acids Res., 8, 2039 (1980)

(18) C. Broka, T. Hozumi, R. Arentzen, and K. Itakura, Nucleic Acids

Res., 8, 5461 (1980).
(19) H. M. Hsiung, W. L. Sung, R. Brousseau, R. Wu, and S. A. Narang, Nucleic Acids Res., 8, 5753 (1980).

(20) M. L. Duckworth, M. J. Gait, P. Goelet, G. F. Hong, and M. Sing,

Nucleic Acids Res., 9, 1691 (1981) (21) P. Dembek, K. Miyoshi, and K. Itakura, J. Am. Chem. Soc., 103, 706

(22) M. D. Mateucci and M. H. Caruthers, J. Am. Chem. Soc., 103, 3185

(1981).

(23) V. Amarnath and A. D. Broom, Chem. Rev., 77, 183 (1977).

(24) C. B. Reese, Tetrahedron, 34, 3143 (1978)

(25) H. Schaller, G. Weiman, B. Lerch, and H. G. Khorana, J. Am. Chem. Soc., 85, 3821 (1963)

(26) H. Büchi and H. G. Khorana, J. Mol. Biol., 72, 251 (1972). (27) B. A. Otter and J. J. Fox, "Synthetic Procedures in Nucleic Acid Chemistry", Vol. 1, W. W. Zorbach and R. S. Tipson, Eds., Interscience Publishers, New York, 1968; p 285.

(28) T. Hata and T. Kurihara, Chem. Lett., 859 (1973). (29) H. Takaku, T. Shimada, Y. Morita, and T. Hata, Chem. Lett., 19 (1976).

(30) A. V. Steinfield, F. Naider, and J. M. Becker, J. Chem. Res. Synop., 129 (1979); J. Chem. Res., Miniprint, 1437 (1979).

(31) J. Igolen and C. Morin, J. Org. Chem., 45, 4802 (1980)

(32) The trimethylchlorosilane used must be carefully stored and handled. Reagent which has been repeatedly exposed to moist air will give unsatisfactory results

Scheme II

with 3b and 3c as well. In each case concentrated aqueous ammonia is added to the reaction mixture to give a 2 M solution. Finally, the N-protected nucleosides 4a-c are obtained by crystallization from water in yields of 95% for 4a and 4b and 75% for 4c from, respectively, 1a-c in about 4 h.

Compounds 4a-c are immediately usable, without further protection, as the hydroxy component in the triester schemes reported by Agarwal<sup>33</sup> and later by Reese<sup>17</sup> for synthesis of diand trinucleoside blocks. The extent of undesired 3',3' joining is quite small and pure products are obtained by chromatography. Although the 5'-O-dimethoxytrityl derivatives of 4a-c may be prepared by known procedures, 25,26 the success of our transient protection approach to synthesis of 4a-c prompted us to explore its use for direct synthesis of the 5'-O-(dimethoxytrityl) derivatives. As shown in Scheme II, the deoxynucleosides are first treated with dimethoxytrityl chloride to give the 5' protected derivatives 5a and 5b. In addition to 5b, deoxycytidine gives a significant and unavoidable amount of the 4-N,5'-O-bis(dimethoxytrityl) compound 5c. However, upon treatment of 5c with trimethylchlorosilane and benzoyl chloride the desired product, 8b, is obtained. Presumably 6c reacts with benzoyl chloride to give a N-benzoyl-N-(dimethoxytrityl) derivative, which would be expected to be unstable and to lose the dimethoxytrityl group. Trimethylchlorosilane alone does not effect cleavage of the N-(dimethoxytrityl) group, nor does addition of anhydrides such as acetic and isobutyric. These anhydrides are considerably less reactive than benzoyl chloride and may be unable to acylate the hindered amino group of 6c. Even using benzoyl chloride, conversion of 6c to 7b is much slower than simple benzoylation of 6b. Nevertheless, treatment of either 5a or the mixture of 5b and 5c, without isolation, gives 8a or 8b in a one-flask procedure, sufficiently pure for immediate phosphorylation. If desired they may be further purified by flash chromatography<sup>34</sup> in overall yields from 1a or 1b of 80-90%. This approach does not work well with deoxyguanosine, which undergoes preferential reaction at N-2

<sup>(33)</sup> K. L. Agarwal and F. Riftina, Nucleic Acids Res., 8, 5491 (1980). (34) W. C. Still, M. Kahn, and A. Mitra, J. Org. Chem., 43, 2293 (1978).

upon treatment with 4,4'-dimethoxytrityl chloride.<sup>25</sup> Moreover, it has become clear that protection of guanine nucleosides is presently inadequate, and that an entirely new concept for the protection of guanine is required. Work along these lines is in progress.

Successful chemical synthesis of oligonucleotides relies on precise manipulation of hydroxy and amino protecting groups. In addition, lipophilic hydroxyl protecting groups have been shown to markedly enhance separation and thereby greatly aid purification of oligonucleotides. Yet the chemical differentiation of nucleoside hydroxyl and amino groups necessary for selective protection has proved surprisingly difficult to achieve. Other than our transient protection approach, only the classic Khorana procedure has allowed such differentiation. Our original goal was only to optimize methods for handling our modified nucleosides. However, the concept of transient protection we developed appears to be widely applicable to a variety of synthetic problems in multifunctional molecules. Some of these applications, principally in nucleoside and nucleotide synthesis, are under investigation in our laboratory and will be reported shortly.

#### **Experimental Section**

General Procedures. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. The nuclear magnetic resonance ( $^1H$  NMR) spectra were recorded on a Varian EM-360 with Me<sub>4</sub>Si as internal reference. Ultraviolet (UV) spectra were recorded on a Cary 118-C spectrophotometer. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, Tenn. Thin-layer chromatography (TLC) was performed on Eastman Chromatogram sheets (silica gel No. 18181, indicator No. 6060) in methylene chloride containing from 1 to 10% methanol, as appropriate. Evaporations were carried out at 40 °C or lower using aspirator or oil pump vacuum.

Flash chromatography<sup>34</sup> was performed on EM silica gel 60, 230–400 mesh. Pyridine was refluxed over and then distilled from calcium hydride and stored over dried 4A molecular sieves. Trimethylchlorosilane, obtained from Aldrich Chemical Co., was handled by syringe and stored in an Aldrich Storage Flask, type C. Deoxynucleosides were purchased from Sigma Chemical Co., Leon Industries, or the United States Biomedical Corp.

6-N-Benzoyl-2'-deoxyadenosine (4a). To 2.5 g (10 mmol) of 1a dried three times by evaporation of pyridine and suspended in 50 mL of dry pyridine was added 6.4 mL (50 mmol) of trimethylchlorosilane. After the mixture was stirred for 15 min 5.8 mL (50 mmol) of benzoyl chloride was added and the reaction was maintained at room temperature for 2 h. The mixture was then cooled in an ice bath and 10 mL of water was added. After 5 min 20 mL of 29% aqueous ammonia was added and the mixture was stirred at room temperature for 0.5 h. The reaction was then evaporated to near dryness and the residue was dissolved in 150 mL of water. The solution was washed once with a 50-mL portion of ethyl acetate. Crystallization began immediately after separation of the layers. After the solution was cooled, filtration gave 3.33 g (94%) of 4a: mp, UV, and NMR were identical with those of material prepared according to the literature. Anal. ( $C_{17}H_{17}N_5O_4$ ,  $^{1/4}H_2O$ ) C, H, N.

**6-N,N-Dibenzoyl-2'-deoxyadenosine.** Reaction was carried out as described above for compound **4a** except that the reaction was quenched by addition of 100 mL of cold saturated NaHCO<sub>3</sub>. The solution was stirred for 4 h and then extracted with  $2 \times 100$  mL portions of ethyl acetate. The combined ethyl acetate layers were evaporated to give the product as a foam in 87% yield. UV<sub>max</sub> (MeOH) 249 mm ( $\epsilon$  20 100), sh 272 ( $\epsilon$  16 600); NMR (CDCl<sub>3</sub>, D<sub>2</sub>O)  $\delta$  8.67 (s, 1, H<sub>8</sub>), 8.38 (s, 1, H<sub>2</sub>), 7.28–8.08 (m, 10, Ar), 6.42 ("t", J = 7 Hz, 1, H<sub>1</sub>), 4.65 (m, 1, H<sub>3</sub>), 4.12 (m, 1, H<sub>4</sub>), 3.80 (br s, 2, H<sub>5',5''</sub>), 2.08–2.88 (m, 2, H<sub>2',2''</sub>). Anal. (C<sub>24</sub>-H<sub>21</sub>N<sub>5</sub>O<sub>5</sub>) C, H, N.

4-N-Benzoyl-2'-deoxycytidine (4b). To 2.27 g (10 mmol) of 1b dried three times by evaporation of pyridine and suspended in 50 mL of dry pyridine was added 6.4 mL (50 mmol) of trimethylchlorosilane.<sup>32</sup> After the solution was stirred 15 min 5.8 mL (50 mmol) of benzoyl chloride was added and the reaction maintained at room temperature for 2 h. The mixture was then cooled in an ice bath and 10 mL of water was added. After 5 min 10 mL of 29% aqueous ammonia was added and the mixture was stirred at room temperature for 15 min. The reaction was then evaporated to near dryness and the residue was dissolved in 150 mL of water. The solution was washed once with a 50-mL portion of ethyl acetate. Crystallization began immediately after separation of the layers.

After the solution was cooled, filtration gave 3.11 g (94%) of **4b**: mp, UV, and NMR were identical with those of material prepared according to the literature. Anal. ( $C_{16}H_{17}N_3O_5$ ) C, H, N.

2-N-Isobutyryl-2'-deoxyguanosine (4c). To 1.34 g (10 mmol of 1c dried three times by evaporation of pyridine and suspended in 50 mL of dry pyridine was added 6.4 mL (50 mmol) of trimethylchlorosilane.32 After the solution was stirred 15 min 8.2 mL (50 mmol) of isobutyric anhydride was added and the solution was maintained at room temperature for 3 h. The reaction was then cooled in an ice bath and 10 mL of water was added. After 5 min 10 mL of 29% aqueous ammonia was added and the reaction was stirred for 15 min. The solution was then evaporated to near dryness and the residue was dissolved in 50 mL of water. The solution was washed once with a 50-mL portion of ethyl acetate:ether (1:1). The organic layer was extracted with a 25-mL portion of water and the combined aqueous layers were concentrated to about 30 mL. Crystallization generally occurred quickly. Concentration of the filtrate gave additional material for a total yield of 2.5 g (75%) of 4c: mp (softens at 130 °C, darkens at 235 °C, does not melt below 300 °C, UV, and NMR were identical with those of material prepared according to the literature.26 Anal. (C<sub>14</sub>H<sub>19</sub>N<sub>5</sub>O<sub>5</sub>·3/<sub>4</sub>H<sub>2</sub>O) C, H, N.

5'-O-(Dimethoxytrityl)-2'-deoxyadenosine (5a). To 2.5 g (10 mmol) of 1a dried three times by evaporation of pyridine and suspended in 50 mL of dry pyridine was added 5 g (15 mmol) of 4,4'-dimethoxytrityl chloride, 2.1 mL (15 mmol) of triethylamine, and 30 mg (0.25 mmol) of 4-dimethylaminopyridine.<sup>36</sup> After 4 h TLC (CH<sub>2</sub>Cl<sub>2</sub>:CH<sub>3</sub>OH, 95:5) showed complete reaction.

The reaction was cooled in an ice bath and 50 mL of 5% aqueous NaHCO<sub>3</sub> was added. The mixture was then extracted with two 150-mL portions of ethyl acetate. The combined organic layers were evaporated to dryness and the residue purified by flash chromatography on silica gel. The appropriate fractions were combined and evaporated and methylene chloride solution of the residue was added dropwise to petroleum ether to precipitate 4.0 g (77%) of 5a: UV<sub>max</sub> (CH<sub>3</sub>OH) 259, 236 nm ( $\epsilon$  17 900, 27 300), UV<sub>min</sub> 254, 225 nm ( $\epsilon$  17 500, 22 700); H NMR (CDCl<sub>3</sub>)  $\delta$  8.22 (s, 1, H<sub>8</sub>), 7.92 (s, 1, H<sub>2</sub>), 7.5–7.0 (m, 9, aryl), 6.9–6.5 (m, 4, aryl), 6.37 (t,  $J_{apparent}$  = 7 Hz, 1, H<sub>1</sub>-), 6.07 (br s, 2, NH<sub>2</sub>), 4.60 (m, 1, H<sub>3</sub>-), 4.12 (m, 1, H<sub>4</sub>-), 3.68 (s, 6, OCH<sub>3</sub>), 3.32 (m, 2, H<sub>5',5''</sub>), 3.1–2.3 (m, 2, H<sub>2',2''</sub>). Anal. (C<sub>31</sub>H<sub>31</sub>N<sub>5</sub>O<sub>5</sub>) C, H, N.

5'-O-(Dimethoxytrityl)-6-N-benzoyl-2'-deoxyadenosine (8a). To the solution obtained at the end of the first paragraph above was added 0.4 mL (50 mmol) of trimethylchlorosilane.<sup>32</sup> After 30 min 5.8 mL (50 mmol) of benzoyl chloride was added and the reaction was stirred at room temperature for 2 h. The mixture was then cooled in an ice bath and 10 mL of water was added. After 5 min 20 mL of 29% aqueous ammonia was added and the mixture was stirred at room temperature for 30 min. The reaction was then evaporated to a gum and the gum partitioned between 150 mL of CH<sub>2</sub>Cl<sub>2</sub> and 150 mL of 5% aqueous NaHCO<sub>3</sub>. The organic layer was evaporated to dryness and the residue purified by flash chromatography on silica gel.<sup>34</sup> The appropriate fractions were combined and evaporated and a methylene chloride solution of the residue was added dropwise to petroleum ether to precipitate 5.5 g (88%) of 8a: UV and NMR were identical with those of material prepared according to the literature.<sup>25</sup> Anal. (C<sub>38</sub>H<sub>35</sub>N<sub>5</sub>O<sub>6</sub>-<sup>2</sup>/<sub>3</sub>H<sub>2</sub>O) C, H, N, O.

5'-O-(Dimethoxytrityl)-2'-deoxycytidine (5b) and 5'-O,4-N-Bis(dimethoxytrityl)-2'-deoxycytidine (5c). To 2.6 g (10 mmol) of 1b dried three times by evaporation of pyridine and suspended in 50 mL of dry pyridine was added 6.7 g (20 mmol) of 4,4'-dimethoxytrityl chloride, 2.8 mL (20 mmol) of triethylamine, and 50 mg (0.42 mmol) of 4-(dimethylamino)pyridine.<sup>36</sup> After 3 h TLC (CH<sub>2</sub>Cl<sub>2</sub>:CH<sub>3</sub>OH, 95:5) showed that no unreacted 1b was present.

The mixture was then poured into 250 mL of cold, saturated NaHCO<sub>3</sub> and the solution was extracted with three 150-mL portions of ethyl acetate. The combined organic layers were evaporated to dryness and the residue purified by flash chromatography on silica gel. <sup>34</sup> Appropriate fractions of the less polar compound were combined and evaporated and a methylene chloride solution of the residue was added dropwise to petroleum ether to precipitate 3.4 g (40%) of 5c: UV<sub>max</sub> (CH<sub>3</sub>OH) 280, 230 nm ( $\epsilon$  18 300, 45 300); UV<sub>min</sub> 259 nm ( $\epsilon$  17 900); NMR (CDCl<sub>3</sub>, D<sub>2</sub>O)  $\delta$  7.58 (d,  $J_{5,6}$  = 8 Hz, 1, H<sub>6</sub>), 7.5-6.5 (m, 26, aryl), 6.32 (t,  $J_{apparent}$  = 7 Hz, 1, H<sub>1</sub>), 4.83 (d,  $J_{5,6}$  = 8 Hz, 1, H<sub>5</sub>), 4.47 (m, 1, H<sub>3</sub>), 4.07 (m, 1, H<sub>4</sub>), 3.75 (s, 12, OCH<sub>3</sub>), 3.33 (m, 2, H<sub>5</sub>; $_{5}$ "), 2.8-1.9 (m, 2, H<sub>2</sub>; $_{2}$ "). Anal. ( $C_{51}H_{49}N_{3}O_{8}$ .  $^{1}/_{2}H_{2}$ O) C, H, N, O.

Appropriate fractions of the more polar compound were combined and evaporated and a methylene chloride solution of the residue was added dropwise to petroleum ether to precipitate 1.7 g (32%) of 5b: UV<sub>max</sub> (CH<sub>3</sub>OH) 274, 234 nm ( $\epsilon$  11 900, 28 800); UV<sub>min</sub> 258 nm ( $\epsilon$  9100); NMR (CDCl<sub>3</sub>, Me<sub>2</sub>SO- $d_6$ , D<sub>2</sub>O)  $\delta$  7.82 (d,  $J_{5,6}$  = 8 Hz, 1, H<sub>6</sub>), 7.5–7.2 (m, 9,

<sup>(35)</sup> R. A. Jones, H.-J. Fritz, and H. G. Khorana, *Biochemistry*, 17, 1268 (1978).

aryl), 7.0–6.7 (m, 4, aryl), 6.17 (t,  $J_{apparent} = 7 \text{ Hz}$ , 1,  $H_{1'}$ ), 5.58 (d,  $J_{5,6} = 8 \text{ Hz}$ , 1,  $H_{5}$ ), 4.40 (m, 1,  $H_{3'}$ ), 4.23 (m, 1,  $H_{4'}$ ), 3.80 (s, 6, OCH<sub>3</sub>), 3.37  $(m, 2, H_{5',5''}), 2.8-1.9 (m, 2, H_{2',2''}).$  Anal.  $(C_{30}H_{31}N_3O_{6'}^{-1}/_2H_2O)$  C, H,

5'-O-(Dimethoxytrityl)-4-N-benzoyl-2'-deoxycytidine (8b). To the solution obtained at the end of the first paragraph above was added 6.4 mL (50 mmol) of trimethylchlorosilane.<sup>32</sup> After 15 min 5.8 mL (50 mmol) of benzoyl chloride was added and the reaction stirred at room temperature for 10 h. The reaction was then cooled in an ice bath and 10 mL of water was added. After 5 min 20 mL of 29% aqueous ammonia was added and the solution stirred at room temperature for 30 min. The mixture was then evaporated to a gum and the gum partitioned between 200 mL of CH<sub>2</sub>Cl<sub>2</sub> and 200 mL of 5% aqueous NaHCO<sub>3</sub>. The aqueous layer was extracted with two 100-mL portions of CH<sub>2</sub>Cl<sub>2</sub>, the combined organic layers evaporated to dryness, and the residue purified

by flash chromatography on silica gel.<sup>34</sup> Appropriate fractions were combined and evaporated and a methylene chloride solution of the residue was added dropwise to petroleum ether to precipitate 5.1 g (81%) of 8b: UV and NMR were identical with those of material prepared according to the literature.25 Anal. (C37H35N3O7) C, H, N.

Acknowledgment. This work was supported by grants from the Biomedical Research Support Grant and the Rutgers Research Council. We thank K. L. Ramachandran, J. Confransico, K. Satra, S. Kuzmich, and B. O'Reilly for technical assistance.

Registry No. 1a, 958-09-8; 1b, 951-77-9; 1c, 961-07-9; 4a, 4546-72-9; 4b, 4836-13-9; 4c, 68892-42-2; 5a, 17331-22-5; 5b, 76512-82-8; 5c, 80594-29-2; 8a, 80594-30-5; 8b, 80594-31-6; 6-N,N-dibenzoyl-2'deoxyadenosine, 6711-37-1.

### Oxidation-Reduction Mechanisms. Inner-Sphere and Outer-Sphere Electron Transfer in the Reduction of Iron(III), Ruthenium(III), and Osmium(III) Complexes by Alkyl Radicals

#### K. L. Rollick and J. K. Kochi\*

Contribution from the Department of Chemistry, Indiana University, Bloomington, Indiana 47405. Received June 4, 1981

Abstract: Alkyl radicals are readily oxidized by the tris(phenanthroline) and tris(bipyridine) complexes ML<sub>3</sub><sup>3+</sup> of iron(III), ruthenium(III), and osmium(III) in acetonitrile solution, the second-order rate constants easily exceeding 106 M<sup>-1</sup> s<sup>-1</sup> at 25 °C. Two oxidative processes are identified as (a) ligand substitution on the coordinated 1,10-phenanthroline to yield various alkylphenanthrolines and (b) cation formation to afford alkenes and N-alkylacetamides (after hydrolysis). Cation formation is characterized by extensive skeletal rearrangement of neopentyl, isobutyl, and n-propyl groups, whereas ligand substitution by the same alkyl radicals occurs without any rearrangement. Steric effects hinder ligand substitution since the rate constant  $k_{\rm L}$  increases in the order neopentyl < isobutyl < n-propyl and  ${\rm Fe}(4,7-{\rm Ph_2phen})_3^{3+}$  <  ${\rm Fe}(4,7-{\rm Me_2phen})_3^{3+}$  <  ${\rm Fe}({\rm phen})_3^{3+}$ . By contrast, cation formation is not subject to steric effects and the rate constant k<sub>R</sub> is invariant for neopentyl, isobutyl, and n-propyl radicals, which all have essentially the same ionization potentials. An outer-sphere mechanism for electron transfer is described for the oxidative process leading to cation formation, in accord with the fit of  $k_{\rm R}$  to the linear free energy relationship established by Marcus theory. An inner-sphere mechanism for the oxidative process leading to phenanthroline substitution is discussed in the context of steric effects on the rate constant  $k_L$  for ligand substitution.

Oxidation-reduction processes mediated by transition-metal complexes are playing an increasing role in organic chemistry. However, the mechanistic distinction in such processes between outer-sphere and inner-sphere electron transfer has not been established, largely owing to inadequately developed criteria.<sup>2</sup> By contrast, electron transfer mechanisms in wholly inorganic systems have been considered for some time,<sup>3</sup> and reasonably reliable experimental and theoretical guidelines have been developed.<sup>4</sup>

The oxidation-reduction of organic free radicals by transition-metal complexes provides an excellent opportunity to examine the mechanism of electron transfer in organic systems, by relying on some of the mechanistic criteria developed in inorganic chemistry. Thus the various alkyl radicals (R.) by their paramagnetic nature are necessarily constrained to undergo oneelectron changes, i.e.<sup>5,6</sup>

Likewise, in the family of coordinatively saturated tris(polypyridine) complexes of the iron triad ML<sub>3</sub><sup>3+</sup>



we have a structurally and chemically homologous series of one-electron oxidants with graded redox potentials. Furthermore, the availability of ligands, especially with L =substituted 1,10phenanthrolines, allows further fine tuning of the redox potentials and the steric properties of these oxidants. 7.8 Coupled with the

<sup>(1) (</sup>a) Wiberg, K. B. Ed. "Oxidation in Organic Chemistry"; Academic Press: New York, 1965. (b) Mayo, F. R., Ed. "Oxidation of Organic Compounds"; American Chemical Society: Washington, D.C., 1968; Vols. 1, 2, and 3. (c) Augustine, R. L., Ed. "Reduction"; Marcel Dekker: New York, 1968. (d) House, H. O. "Modern Synthetic Reactions", 2nd ed.; W. A. Benjamin: Menlo Park, Calif., 1972.

A. benjamin: Menio Park, Calif., 19/2.

(2) Littler, J. S. Spec. Chem. Publ.—Soc. 1970, 24, 383. See, however: Ng, F. T. T.; Henry, P. M. J. Am. Chem. Soc. 1976, 98, 3606.

(3) Taube, H. Adv. Inorg. Radiochem. 1959, I, 1. Halpern, J. Q. Rev., Chem. Soc. 1961, 15, 207.

(4) (a) Reynolds, W. L.; Lumry, R. W. "Mechanisms of Electron Transfer"; Ronald Press: New York, 1966. (b) Sutin, N. In "Inorganic Biochemistry": Eichhorn, G. L. Ed. Elsevier: Amsterdam, 1973, Vol. 2, p. Biochemistry"; Eichhorn, G. L., Ed., Elsevier: Amsterdam, 1973; Vol. 2, p 611. (c) Cannon, R. D. "Electron Transfer Reactions"; Butterworths: Boston, 1980. (d) Pennington, D. E. ACS Monogr. 1978, No. 174, 476.

<sup>(5) (</sup>a) Littler, J. S.; Nonhebel, D. C. Int. Rev. Sci.: Org. Chem., Ser. Two 1975, 10, 21. (b) Kochi, J. K. "Free Radicals"; Wiley: New York, 1973; Vol. 1, p 591 ff.

<sup>(6)</sup> Walling, C. Acc. Chem. Res. 1975, 8, 125.

# EXHIBIT 19

# CARBOHYDRATE RESEARCH PHYSICAL SCIENCE MAR 13 1978

LIERARY.

## AN INTERNATIONAL JOURNAL

#### REGIONAL EDITORS

- A. B. FOSTER (London)
- D. Horton (Columbus, Ohio)
- R. W. JEANLOZ (Boston, Mass.)
- R. STUART TIPSON (Kensington, Md.)
- J. M. WEBBER (Birmingham)

#### EDITORIAL ADVISORY BOARD

- L. Anderson (Madison, Wisc.)
- S. J. ANGYAL (Sydney)
- G. O. ASPINALL (Downsview, Ont.)
- H. H. BAER (Ottawa)
- J. N. BEMILLER (Carbondale, Ill.)
- J. S. Brimacombe (Dundee)
- J. G. BUCHANAN (Edinburgh)
- B. CASU (Milan)
- M. ČERNÝ (Prague)
- S. DAVID (Orsay)
- J. DEFAYE (Grenoble)
- J. O. DEFERRARI (Buenos Aires)
- H. EL KHADEM (Houghton, Mich.)
- R. J. FERRIER (Wellington)
- D. FRENCH (Ames, Iowa)
- A. GÓMEZ-SÁNCHEZ (Seville)
- L. GOODMAN (Kingston, R. I.)
- L. D. HALL (Vancouver)
- S. HANESSIAN (Montreal)
- K. HEYNS (Hamburg)
- J. E. Hodge (Peoria, Ill.)

- L. Hough (London)
- H. S. ISBELL (Washington, D.C.)
- J. F. KENNEDY (Birmingham, U.K.)
- N. K. KOCHETKOV (Moscow)
- J. LEHMANN (Freiburg)
- F. W. LICHTENTHALER (Darmstadt)
- B. LINDBERG (Stockholm)
- P. F. LLOYD (Bangor)
- D. J. MANNERS (Edinburgh)
- F. MICHEEL (Münster)
- R. MONTGOMERY (Iowa City, Iowa)
- J. Montreuil (Villeneuve d'Asca)
- K. ONODERA (Kyoto)
- T. OSAWA (Tokyo)
- W. G. OVEREND (London)
- H. PAULSEN (Hamburg)
- C. PEDERSEN (Lyngby)
- A. S. PERLIN (Montreal)
- D. A. REES (Sharnbrook)
- G. N. RICHARDS (Townsville)
- L. RODÉN (Birmingham, Ala.)
- N. SHARON (Rehovoth)
- A. M. STEPHEN (Cape Town)
- L. SZABÓ (Orsav)
- W. A. SZAREK (Kingston)
- O. THEANDER (Uppsala)
- J. M. J. TRONCHET (Geneva)
- S. UMEZAWA (Yokohama)
- R. L. WHISTLER (Lafayette, Ind.)
- A. ZAMOJSKI (Warsaw)
- Yu. ZHDANOV (Rostov-on-Don)

Carbohydrate Research 60 (1978) 206-209

© Elsevier Scientific Publishing Company, Amsterdam - Printed in The Netherlands

#### Note

#### Facile detritylation of nucleoside derivatives by using trifluoroacetic acid\*

MALCOLM MACCOSS AND DANIEL J. CAMERON

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439 (U.S.A.)

(Received April 25th, 1977; accepted for publication, May 18th, 1977)

The triphenylmethyl (trityl) ether is one of the most commonly used, acidlabile protecting groups for primary hydroxyl functions in carbohydrate and nucleoside chemistry<sup>1,2</sup>. Previous methods for deprotection have included 80% acetic acid at reflux<sup>3</sup>, hydrogen bromide in acetic acid<sup>4</sup>, hydrogen chloride in various organic solvents<sup>5-7</sup>, sodium in liquid ammonia<sup>8</sup>, catalytic hydrogenation<sup>3,9</sup>, and absorption onto silica gel<sup>10</sup>.

On using these procedures, difficulties have arisen in certain instances; for example, detritylation of 2,3'-anhydro-1-(2,5-di-O-trityl- $\beta$ -D-xylofuranosyl)uracil (1) with hydrogen chloride in ethanol to yield 2,3'-anhydro-1-( $\beta$ -D-xylofuranosyl) uracil<sup>7</sup> gives as much as 34% of 1-(5-chloro-5-deoxy- $\beta$ -D-xylofuranosyl)uracil as a by-product, because of a rearrangement of the anhydro ring, followed by nucleophilic opening by chloride ion<sup>11-12</sup>. In the hydrogenolysis of trityl derivatives of nucleosides, the reaction has been shown to be somewhat sluggish and unsatisfactory<sup>13</sup>.

<sup>\*</sup>This work was supported by the U.S. Energy Research and Development Administration.

207

The main drawback to the use of the trityl protecting group has been with purine deoxynucleosides, where the glycosyl bond is especially susceptible to acid hydrolysis, and concomitant cleavage of the base usually occurs<sup>14</sup>. The presence of an electron-withdrawing group on the sugar has been shown to stabilize the glycosyl bond to acid hydrolysis<sup>15,16</sup>, and detritylation without excessive glycosyl cleavage has been found possible for purine deoxynucleosides having 3'-O-trifluoroacetyl15. 3'-O-acetyl16,17, or 3'-O-tosyl substituents17. Usually, it has been necessary to resort to the more-labile mono-(p-methoxyphenyl)diphenylmethyl or di-(p-methoxyphenyl) phenylmethyl ethers to permit deprotection without cleavage of the base<sup>14</sup>. Benzenesulfonic acid has recently been shown to be effective for deblocking such derivatives 18. However, these substituted trityl groups usually show lower selectivity for the primary hydroxyl group during their introduction<sup>2</sup>.

A mixture of 1-butanol-formic acid-toluene has been used to deprotect various "methoxytrityl"-protected, aminoacyl nucleoside derivatives19, and aqueous trifluoroacetic acid (TFA) has been used for the hydrolysis of sugar acetals<sup>20</sup>. This report describes the use of 1-butanol-TFA for hydrolyzing trityl ethers under exceptionally mild conditions.

In our initial studies to find a better procedure, the detritylation of 1 was accomplished with 90% (v/v) aqueous TFA for 2 min at room temperature to yield 2,3'-anhydro-1-( $\beta$ -D-xylofuranosyl)uracil<sup>7</sup> as the sole u.v.-absorbing product. This result is in direct contrast to previous procedures<sup>7,12</sup>, which gave rise to various proportions of by-products. A simple isolation (see Experimental) gave 67% of the crystalline deprotected product, with no attempt being made to reprocess the mother liquors. When these conditions were applied to 2'-deoxy-5'-O-trityladenosine (2), excessive glycosyl-bond cleavage occurred. This could be minimized by using TFA diluted with 1-butanol and by terminating the hydrolysis by rapid neutralization of the mixture with Bio-Rad  $1 \times 2$  (OH $^-$ ) resin prior to evaporation of volatile materials. An overall yield of 79 % of 2'-deoxyadenosine (sole u.v.-absorbing product) could be obtained from 2 by this procedure, thus demonstrating the first detritylation of an unsubstituted purine deoxyribonucleoside in good yield. It should be noted that any adenine formed during this hydrolysis would be retained on the resin.

The use of the ion-exchange resin to effect rapid neutralization of the mixture was not necessary for the deprotection of 5'-O-trityladenosine (3), 5'-O-trityluridine (4), and 5'-O-tritylthymidine (5), all of which are more stable to glycosyl bondcleavage under acid conditions. In these instances, the mixtures were processed by dilution with 1-butanol and evaporation of volatile products in vacuo, followed by partition between water and ether to yield the chromatographically pure, deprotected nucleoside in the aqueous layer. When these procedures were used for deprotecting 2, various proportions of adenine were produced. The mode of isolation was shown, by subjecting 3 to two parallel reactions, to influence the time needed for reaction. In one instance, the reaction was terminated by evaporation of volatile materials, and in the other it was terminated after the same length of time by rapid neutralization with Bio-Rad 1 × 2 (OH<sup>-</sup>) resin. The former gave a quantitative

TABLE I

DETRITYLATION WITH 1-BUTANOL-TRIFLUOROACETIC ACID

Starting material (rej	Product <sup>a</sup> (.)	1-Butanol–TFA (by volume)	Time (min)	Yield <sup>b</sup> (%)
1 (7)	2,3'-anhydro-1-(β-D- xylofuranosyl)uracil	9:10	2	67ª
2 (13)	2'-deoxyadenosine	1.86:1	3	79
3€	adenosine	3:1	30	100
4e	uridine	3:1	30	100
5e	thymidine	3:1	30	100
6 (17)	3'-O-acetyl-2'-deoxyadenosine	3:1	30	93 <i>f</i>

<sup>&</sup>lt;sup>a</sup>Compared with authentic samples. <sup>b</sup>Estimated by u.v., purity checked by t.l.c. (see Experimental). <sup>c</sup>H<sub>2</sub>O-TFA. <sup>a</sup>Crystallized yield (from ethanol). <sup>e</sup>Purchased from Sigma. <sup>f</sup>Product contained ~5% adenine.

yield of adenosine, but the latter yielded  $\sim 62\%$  of unreacted 3. This difference is presumably due to concentration of the acid and/or the continuation of the reaction during the evaporation process.

The increased stability of the glycosyl bond in 3'-O-acetyl-2'-deoxy-5'-O-trityladenosine (6) relative to 2 (refs. 16 and 17) was again demonstrated by the fact that, in the case of 6, the reaction could be terminated by evaporation of volatiles with the accompaniment of only a small extent ( $\sim 5\%$ ) of glycosyl bond-cleavage. The optimum conditions and yields for 1-6 are shown in Table I.

Finally, subjecting 2',3'-O-isopropylideneuridine to the same conditions as used for the detritylation of 3-6 showed (t.l.c.) no deblocking, indicating that, if desired, the selective removal of a trityl group in the presence of an isopropylidene group should be feasible. Also, the dinucleoside monophosphate, uridylyl- $(3' \rightarrow 5')$ -uridine (UpU), was treated with 90% aqueous TFA under the identical conditions that caused complete deblocking of 1. Examination of the crude product by n.m.r. spectroscopy (220 MHz) showed no detectable isomerization of the internucleotide linkage<sup>21,22</sup>.

#### **EXPERIMENTAL**

General procedure. — The tritylated nucleoside was dissolved in the appropriate solution of trifluoroacetic acid (see Table I) to a concentration of 1–10 mm and kept for the time indicated at room temperature. The reaction was monitored by t.l.c. [Eastman Silica Gel sheets no. 13181 with either (A) the upper phase of 4:1:2 (by volume) ethyl acetate-1-propanol-water, or (B) 1:49 (by volume) methanol-chloroform, as developing solvent]. The reaction was terminated by the addition of 1-butanol (2 vols) and evaporation to dryness in vacuo (temp. <40°). This residue was separated between water and ether (washing the aqueous layer well with ether) to yield the deprotected nucleoside as the sole component in the aqueous layer.

For compound 2, a sample (0.028 mmol) in 9 ml of 1-butanol-TFA (1.86:1) was treated by the foregoing procedure and the reaction was rapidly terminated after 3 min by addition of the mixture to a cooled  $(-10^{\circ})$ , well-stirred suspension of Bio-Rad 1 × 2 (OH<sup>-</sup>) resin (120 ml) in methanol. The resin was filtered off, washed well with methanol, and the filtrate evaporated to dryness. This residue was separated between water (25 ml) and ether (25 ml), and the aqueous layer was washed with ether (2 × 50 ml) to yield 2'-deoxyadenosine (0.022 mmol; 79%) in the aqueous phase, as the sole u.v.-absorbing product.

Glycosyl bond-cleavage was evaluated in all cases by t.l.c. [System A for 1, 4-6 and Cellulose sheets no. 13254, with 4:3:2:1 (by volume) tert-butanol-butan-2-one water-ammonium hydroxide as developing solvent, for 2 and 3.]

#### REFERENCES

- 1 B. Helferich, Adv. Carbohydr. Chem., 3 (1948) 79-111.
- 2 C. A. Dekker and L. Goodman, in W. Pigman and D. Horton (Eds.), The Carbohydrates, Chemistry and Biochemistry, Vol. IIA, Academic Press, New York and London, 1970, pp. 22-28.
- 3 F. MICHEEL, Ber., 65 (1932) 262-265.
- 4 B. HELFERICH AND W. KLEIN, Ann., 450 (1926) 219-229.
- 5 Y. M. CHOY AND A. M. UNRAU, Carbohydr. Res., 17 (1971) 439-443.
- 6 P. E. VERKADE, J. VAN DER LEE, AND W. MEERBURG, Rec. Trav. Chim., 54 (1935) 716-724.
- 7 N. C. YUNG AND J. J. FOX, J. Am. Chem. Soc., 83 (1961) 3060-3066.
- 8 P. Kováč and S. Bauer, Tetrahedron Lett., (1972) 2349-2350.
- 9 P. E. VERKADE, W. D. COHEN, AND A. K. VROEGE, Rec. Trav. Chim., 59 (1940) 1123-1140.
- 10 J. Lehrfeld, J. Org. Chem., 32 (1967) 2544-2546.
- 11 K. KIKUGAWA AND T. UKITA, Chem. Pharm. Bull. (Tokyo), 17 (1969) 775-784.
- 12 K. KIKUGAWA, M. ICHINO, AND T. UKITA, Chem. Pharm. Bull. (Tokyo), 17 (1969) 785-797.
- 13 W. Anderson, D. H. Hayes, A. M. Michelson, and A. R. Todd, J. Chem. Soc., (1954) 1882-1887.
- 14 H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, J. Am. Chem. Soc., 85 (1963) 3821-3827.
- 15 M. J. ROBINS AND G. L. BASOM, Can. J. Chem., 51 (1973) 3161-3169.
- 16 H. P. C. HOGENKAMP AND T. G. OIKAWA, J. Biol. Chem., 239 (1964) 1911-1916.
- 17 M. J. ROBINS, J. R. McCarthy, Jr., and R. K. Robins, Biochemistry, 5 (1966) 224-231.
- 18 J. STAWINSKI, T. HOZUMI, S. A. NARANG, C. P. BAHL, AND R. WU, Nucleic Acids Res., 4 (1977) 353-371.
- 19 M. J. ROBINS, R. A. JONES, AND M. MACCOSS, Biochemistry, 13 (1974) 553-559.
- 20 J. E. CHRISTENSEN AND L. GOODMAN, Carbohydr. Res., 7 (1968) 510-512.
- 21 C. H. LEE, F. S. EZRA, N. S. KONDO, R. H. SARMA, AND S. S. DANYLUK, Biochemistry, 15 (1976) 3627-3637.
- 22 F. S. Ezra, N. S. Kondo, C. F. Ainsworth, and S. S. Danyluk, Nucleic Acids Res., 3 (1976) 2549-2562.

# EXHIBIT 20

## IN THE UNITED STATES DISTRICT COURT FOR THE DISTRICT OF DELAWARE

NIPPON SHINYAKU CO., LTD.,

Plaintiff,

v.

SAREPTA THERAPEUTICS, INC.,

Defendant.

C.A. No. 21-1015 (GBW)

SAREPTA THERAPEUTICS, INC.,

Defendant/Counter-Plaintiff,

v.

NIPPON SHINYAKU CO., LTD. and NS PHARMA, INC.

Plaintiff/Counter-Defendants.

REPLY DECLARATION OF DR. BRADLEY L. PENTELUTE

### **Table of Contents**

				<u>Page</u>		
I.	INTF	RODUC	CTION	1		
II.	TAS	TASK SUMMARY AND MATERIALS CONSIDERED				
III.	RESI	RESPONSE TO DR. LUEDTKE'S BACKGROUND INFORMATION				
	A.	Skille	ed Artisan	2		
	B.	NS's	s '322 Patent	3		
IV.	DISPUTED CLAIM TERMS OF NS'S '322 Patent					
	A.		e): "reacting said Compound 3 with a deprotecting agent to form pound 4"	8		
		1.	A Skilled Artisan Would Have Understood that the Claimed Steps, Including Step e), Must Be Performed in the Order Written	8		
			a. Dr. Luedtke Bases His Opinions on a Limitation Not in the Claims	8		
			b. Dr. Luedtke's Hypothetical Reactions Inserted Between Steps d) and e) Are Irrelevant and Undesirable	10		
		2.	A Skilled Artisan Would Have Understood that Compound 3 Directly Reacts with a Deprotecting Agent	12		
		3.	NS's Construction Is Inconsistent with a Skilled Artisan's Understanding	18		
	B.		f): "reacting Compound 4 with an acid to form said oligomer" or eting said Compound 4 with an acid to form said PMO"	19		
		1.	A Skilled Artisan Would Have Understood that the Claimed Steps, Including Step f), Must Be Performed in the Order Written	19		
			a. Dr. Luedtke Bases His Opinions on a Limitation Not in the Claims	19		
			b. Dr. Luedtke's Hypothetical Reactions Inserted Between Steps e) and f) Are Irrelevant and Undesirable	21		
		2.	A Skilled Artisan Would Have Understood that Compound 4 Directly Reacts with an Acid	23		

 I, Bradley L. Pentelute, Ph.D., declare as follows:

#### I. INTRODUCTION

- 1. I have been retained by Sarepta Therapeutics, Inc. ("Sarepta") as an independent expert in the synthesis of antisense oligonucleotides. I understand that Nippon Shinyaku Co., Ltd. and NS Pharma, Inc. ("NS") have asserted claims 1-10 of U.S. Patent No. 10,683,322 ("the '322 patent"; Ex. 2) against Sarepta. I submit this declaration on behalf of Sarepta in response to the Declaration of Nathan W. Luedtke, Ph.D., dated February 27, 2023 ("Luedtke Decl.").
- 2. My prior Declaration ("Pentelute Decl."), dated February 5, 2023, is incorporated by reference in its entirety. In it, I have provided my qualifications, compensation and prior testimony, and background information in Sections II, III, and IV, respectively. My curriculum vitae is attached to my prior Declaration at **Appendix A**.

#### II. TASK SUMMARY AND MATERIALS CONSIDERED

3. I have considered the Luedtke Declaration and its cited exhibits. I have also considered the materials cited in this declaration, including those listed in **Appendix C**. To the extent I am provided with additional information, including any expert declaration(s) in this case, I reserve the right to modify, supplement, and/or expand my opinions based on that information or any additional information that may be relevant to the case. I also reserve the right to prepare slides or other demonstratives to help illustrate my opinions.

#### III. RESPONSE TO DR. LUEDTKE'S BACKGROUND INFORMATION

#### A. Skilled Artisan<sup>1</sup>

- 4. Dr. Luedtke does not fully agree with the definition of a skilled artisan proposed in my prior Declaration. Luedtke Decl. ¶22. While Dr. Luedtke agrees that an individual with a Ph.D. would qualify as a skilled artisan with respect to the '322 patent, he proposes that a skilled artisan could alternately have a B.S./B.A. or M.S. *Id.* ¶¶22–23. Dr. Luedtke also disagrees that a skilled artisan must have "specific experience with the use of antisense oligonucleotides for inducing exon skipping and/or testing the safety and efficacy of oligonucleotides." *Id.* ¶22. I disagree and maintain that the definition of a skilled artisan proposed in my prior Declaration is correct. *See* Pentelute Decl. ¶¶21–22.
- 5. First, I disagree that a person with only a B.S./B.A. or M.S. would qualify as a skilled artisan. As I previously summarized, the subject matter of the '322 patent is a multistep chemical synthesis (involving over 50 steps). *Id.* ¶¶23–39. Understanding the chemistry involved in those reaction steps, as well as the technical background involved, requires years of graduate education and research experience in broad areas including organic chemistry, physical organic chemistry, solid phase synthesis, oligonucleotide chemistry, chemical biology, and a B.S./B.A. or M.S. degree would not provide the necessary education and experience.
- 6. Second, I disagree that experience with testing and/or using antisense oligonucleotides is not required. The claims are directed to methods of making phosphorodiamidate morpholino oligomers (PMOs) that are 100% complementary to a region of

2

<sup>&</sup>lt;sup>1</sup> It appears that Dr. Luedtke uses the term "person of ordinary skill in the art" or "POSA" as a synonym for the term "skilled artisan" used in my prior Declaration and in this declaration. *See* Luedtke Decl. ¶18-23.

the human dystrophin pre-mRNA that "hybridize[] to" that pre-mRNA by "Watson-Crick base pairing." Ex. 2 at claim 1. A skilled artisan making PMOs in accordance with the claimed methods of the '322 patent must also be able to test and confirm that the PMOs hybridize to the designated region within the human dystrophin pre-mRNA. Further, as the '322 patent states, the goal of making antisense oligonucleotides is to test and use them as therapies. *Id.* at Abstract ("Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency."). Achieving that goal requires expertise in making, testing, and using antisense oligonucleotides as therapies. Thus, the skilled artisan would ultimately need to have both training in the synthesis of antisense oligonucleotides as well as experience with testing and/or using antisense oligonucleotides.

7. Despite our disagreement over the definition of a skilled artisan, the opinions offered in my previous Declaration and in this Declaration would not change under either Dr. Luedtke's or my definition.

#### B. NS's '322 Patent

- 8. As I previously explained, each of claims 1 and 6 of the '322 patent recites a solid-phase method of making a PMO. Pentelute Decl. ¶13–15. This method is stepwise, involving six separate steps labeled as step a) through step f) in alphabetical order. *Id.* Step a) provides Compound 1, a first morpholino monomer linked to a solid carrier. *Id.* Each of steps b) through f) recites a particular chemical reaction, reacting "said Compound" from the prior step with a reagent (e.g., base, acid, deprotecting agent) to form another sequentially numbered "Compound" used in the next step (or the final product, the oligomer or PMO). *Id.* ¶42–44. The compounds made and used in these steps are numerically identified from Compound 1 to Compound 4. *Id.*
- 9. In his declaration, Dr. Luedtke offers his characterization of the solid-phase method of the '322 patent. Luedtke Decl. ¶26–33. According to Dr. Luedtke:

- each reference to "said Compound" means "a Compound having the previously defined chemical structure" (id. ¶¶29-30) (emphasis added);
- so-called "reversible chemical reactions" could theoretically take place between the recited steps, yet still be covered by the claims (*id.* ¶¶31–32); and
- while steps a) through d) must be performed in the listed order, steps e) and f) can be performed in "any order" (id. ¶¶31–33).

As explained below, I respectfully disagree with each of these points.

- Oligomer, a skilled artisan must perform a linear series of steps a) through f), making and using Compounds 1 through 4 sequentially. Each step that uses the term "said Compound" refers back to *the* same numbered Compound resulting from the previous step. Pentelute Decl. ¶42–45. The specification's sole synthesis scheme conforms to this reading of the claims. Like the steps recited in the claims, each reaction step in that scheme uses a numbered Compound "produced in" the previous step to generate the next sequentially numbered Compound used in the next step (or in the case of step f), the final product). *Id.* ¶46–51.
- 11. Dr. Luedtke's interpretation of "said Compound" as "a Compound having the previously defined chemical structure" conflicts with the stepwise logic of the claims and example in the specification. As Dr. Luedtke admits, his interpretation transforms the term "said Compound" to a Compound from any source obtained by any method. See Luedtke Decl. ¶¶54, 76. But the claims expressly instruct a skilled artisan to use "said Compound" provided in the prior step, not a Compound obtained from a different source. The specification provides the same instruction, repeatedly instructing a skilled artisan to use the Compound "produced in" a prior step. The specification does not discuss other sources or methods for obtaining the claimed Compounds,

and Dr. Luedtke likewise does not point to any specific disclosures in the specification to support his position. *See id.* ¶¶26–33.

- 12. Dr. Luedtke premises his construction on his interpretation on what the word "said" means in the context of patent law. Specifically, Dr. Luedtke states that the word "said" in patent law refers back to an earlier phrase introduced in the claims and when the word "said" is used, the scope of the associated claim term has the same scope as the earlier phrase. *See id.* ¶29. Even if that is true, Dr. Luedtke's interpretation is still inconsistent with the claims, which define each "Compound" both by how it is made and by its structure.
  - 13. Step b), which is reproduced below, is illustrative.

- 14. The text of step b) explains that "Compound 2" is formed by reacting Compound 1 with an acid. The associated drawing depicts a defined chemical formula for the product of this reaction, Compound 2. This information conveys to a skilled artisan that "Compound 2" refers to the specific compound made by the particular chemical reaction recited in that step.
- 15. The same applies to both "Compound 3" and "Compound 4." Based on the plain language of step d), a skilled artisan would have understood that "Compound 3" is formed by "repeating steps b) and c)" to produce a compound having a particular chemical formula. Similarly, based on the plain language of step e), a skilled artisan would have understood that

"Compound 4" refers to the specific compound made by "reacting said Compound 3 with a deprotecting agent" to produce a compound having a particular chemical formula.

- 16. Given that each of these steps defines the "Compound" both by its structure and the method used to synthesize it, a skilled artisan would have understood that each reference to "said Compound" in the claims means *the* Compound made and depicted in the prior step. This is also consistent with how the specification defines each Compound, i.e., by its structure *and* the step used to make it. *See*, *e.g.*, Ex. 2 at 22:7–67 ("Compound (VII) *produced in* Step B"). As such, a skilled artisan would understand that the term "said Compound" means the Compound from the previous step, not, as Dr. Luedtke contends, a Compound from *any* source made by *any* method.
- 17. Reversible chemical reactions: Dr. Luedtke argues that a skilled artisan "could" perform reversible chemical reactions, converting, for example, Compound 2 made in step b) to another compound and then reverting that new compound back to Compound 2. Luedtke Decl. ¶32. According to Dr. Luedtke, the skilled artisan could then continue the remaining recited steps using that new Compound 2. *Id*. Consistent with the overall logic of the synthesis scheme, however, the plain language of the recited steps is clear: the first step of the claimed methods, step a), "provid[es] Compound 1," and each step that follows step a) uses "said Compound" to carry out another reaction. The claims do not suggest a series of additional chemical reactions reversibly creating an additional, unrecited compound between the recited steps.
- 18. Dr. Luedtke's hypothesis is also inconsistent with the research experience of a skilled artisan. In general, scientists avoid unnecessary and wasteful reactions because they decrease yield and can negatively affect the impurity profile and stereochemistry.<sup>2</sup> This is

6

<sup>&</sup>lt;sup>2</sup> Atoms in each chemical compound have a certain relative spatial arrangement, generally referred to as the "stereochemical" property of the compound. Chemical reactions can disturb this arrangement, causing atoms to assume a different special arrangement after the reactions.

certainly true in synthesizing antisense oligomers, which are made via a series multiple steps. Each additional step can produce unwanted impurities, which in aggregate can significantly complicate the synthesis as a whole and impact overall yield and purity. Given the complexity, a skilled artisan would not have deviated from the stepwise method set forth in the claims. A skilled artisan would not consider Dr. Luedtke's hypothetical reversible reactions desirable, let alone permitted in view of the plain claim language.

- 19. Order of steps: Dr. Luedtke asserts that steps a) through d) must be performed in the listed order, but steps e) and f) could be performed in any order. Luedtke Decl. ¶33. But the claims use consistent language and nomenclature throughout: the steps are all alphabetically labeled from a) through f), and the compounds made and used in those steps are numerically ordered from Compound 1 through Compound 4. A skilled artisan reading the claims would not distinguish steps e) and f) from the preceding steps in terms of their required order. Rather, a skilled artisan would read the claims as requiring the specified order of steps from the beginning (step a)) to the end (step f)) of the claimed method.
- 20. Consistent with the claims, the specification similarly treats all of the steps equally: both the generic synthesis scheme and the sole synthesis example in the specification follow the *exact* order of steps recited in the claims, i.e., listing and performing steps a) through f) in order. Pentelute Decl. ¶¶46–51. Nothing in the claims or the specification indicates that only steps a) through d) must be performed in order. To the contrary, the patent teaches that all of the steps, including steps e) and f), must be performed in the listed order.
- 21. Dr. Luedtke states that unlike steps a) through d), steps e) and f) can be performed out of order because they are "polishing or finishing steps," which can vary based on "the purification methods available to" the skilled artisan. Luedtke Decl. ¶33. Regardless of what

purification methods a skilled artisan may or may not have access to, the logic and plain language of the claims and specification make clear that all of the steps, including steps e) and f), must be performed in the order written.

22. Furthermore, Dr. Luedtke's characterization of steps e) and f) as "polishing or finishing steps" improperly trivializes these steps. In the context of the claimed methods, each of steps e) and f) are essential for properly deprotecting the assembled PMO at multiple chemically reactive sites and cleaving it from the solid carrier. Taking these steps out of sequence could lead to a product that is incorrectly deprotected, still attached to the solid carrier, and/or having an altered impurity profile or reduced yield, all of which could render the product unsuitable and no longer functional. Dr. Luedtke's characterization of steps e) and f) as trivial and his suggestion that those steps therefore can be performed out of order do not conform to a skilled artisan's understanding or experience in the field.

#### IV. DISPUTED CLAIM TERMS OF NS'S '322 PATENT

### A. Step e): "reacting said Compound 3 with a deprotecting agent to form Compound 4"

Term	NS's Position	Sarepta's Position
"e) reacting said	Plain and ordinary meaning—i.e.,	Plain and ordinary meaning, i.e.,
Compound 3 with a	chemically reacting Compound 3	chemically reacting a deprotecting
deprotecting agent	with a deprotecting agent, in order	agent directly with Compound 3 of
to form Compound	to form Compound 4	step d), which results in
4"		Compound 4

- 1. A Skilled Artisan Would Have Understood that the Claimed Steps, Including Step e), Must Be Performed in the Order Written
  - a. Dr. Luedtke Bases His Opinions on a Limitation Not in the Claims
- 23. As discussed previously, a skilled artisan would have understood that the steps of the claims, including step e), must be performed in the order specified in the claims. Pentelute Decl. ¶¶40–51. The claimed synthesis methods require performing six steps, alphabetically

ordered from a) through f), and making and using Compounds numerically ordered from Compound 1 through Compound 4. The claims expressly link these steps in the listed order, requiring each step to use "said" Compound made in the prior step and form another Compound used in the next step. Both the generic synthesis scheme and synthesis example in the specification follow this listed order and instruct a skilled artisan to use a Compound "produced in" the prior step. Read in this context, a skilled artisan would have concluded that the reaction of step e) must use Compound 3 from step d) and result in Compound 4, which is then used in the next step.

- 24. Dr. Luedtke in response disagrees with Sarepta's construction, contending that step e) does not require using Compound 3 produced from step d). Luedtke Decl. ¶¶50–62. But his disagreement is premised on the same flawed interpretation addressed above, i.e., that "said Compound 3" refers to "a Compound 3 having the same previously defined chemical structure and does not require Compound 3 to come from any specific source." Id. ¶54 (emphasis added).
- 25. As discussed above, I disagree. *See supra* ¶10–16. Dr. Luedtke's interpretation converting "said Compound 3" to "a Compound 3" from "any" source is inconsistent with both the claims and the specification. Step e) recites using "said Compound 3" from the previous step, not "a Compound 3" obtained from a different source. *See* Ex. 2 at step e) of claims 1 and 6. Further, the specification expressly instructs a skilled artisan to use Compound 3 "produced in" the previous step, not some other, undefined step. *Id.* at 22:7–67 ("Compound (VII) *produced in* Step B"). The specification does not discuss any other method for obtaining Compound 3. By changing the claim term "said Compound 3" to the phrase "a Compound 3," Dr. Luedtke adds a limitation that does not exist in the claims.
- 26. Dr. Luedtke's reasoning—that "said Compound 3" refers to the previously defined structure only—is similarly flawed, as discussed above. Luedtke Decl. ¶53; see supra ¶¶10–16.

A shown below, the text of step d) requires that "Compound 3" is formed by repeating steps b) and c) and the associated drawing depicts a defined chemical formula for the product, Compound 3:

A skilled artisan would have concluded from this express language and chemical structure that the claim term "said Compound 3" in step e) refers to the specific compound made by the particular chemical reaction in step d).

- b. Dr. Luedtke's Hypothetical Reactions Inserted Between Stepsd) and e) Are Irrelevant and Undesirable
- 27. In support of his construction, Dr. Luedtke states that certain "unrecited steps could be performed between steps d) and e) that *could* alter the result of step d)." Luedtke Decl. ¶55 (emphasis added). Dr. Luedtke then identifies two types of hypothetical reactions that, in his view, can be performed between steps d) and e). *Id.* ¶¶56–62. I disagree.
- 28. As an initial matter, the plain language of the claims prohibits additional steps that alter the chemical structures of the recited Compounds. Step e) expressly requires using "said Compound 3"—the Compound 3 made in step d). Nothing in the claims or specification suggests performing additional, unrecited reactions that chemically transform Compound 3 from step d) to

another, undefined compound. Indeed, the only sequence of reactions that the specification discloses includes step d) immediately followed by step e). *See* Pentelute Decl. ¶¶46–51; Ex. 2 at 19:21–26 ("repeating step A and step B of the PMO production method described in the specification for a desired number of times"); id. at 22:7–67 (Step C: "In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX)."). A skilled artisan would not have understood that reactions "alter[ing] the result of step d)" (Luedtke Decl. ¶55) between steps d) and e) are permitted.

- Turning to specifics of Dr. Luedtke's hypothetical reactions, I disagree that a skilled artisan would have performed the proposed reactions between steps d) and e). He first proposes the same type of reversible chemical reactions addressed above, e.g., (1) converting Compound 3 from step d) to another compound (e.g., removing the trityl group from Compound 3) and then (2) reverting back to Compound 3 (e.g., re-adding the trityl group). Luedtke Decl. ¶58–59; see supra ¶17–18. Dr. Luedtke does not provide any reason for why a skilled artisan would perform these unnecessary reactions, which would decrease yield and risk materially changing important aspects of Compound 3. See Luedtke Decl. ¶58–59; see supra ¶17–18. In my opinion, a skilled artisan would not have considered these reversible chemical reactions desirable or permitted.
- 30. The second type of reaction that Dr. Luedtke proposes is a linker modification, for example, switching from one linker to a different, known linker called a "safety-catch" linker. Luedtke Decl. ¶60. Similar to the reversible chemical reactions above, a skilled artisan would not have any reason to switch the linker midstream during PMO synthesis, which could chemically damage the assembled PMO monomers or cause complete product loss. Reinforcing the speculative nature of this hypothetical, the two references that Dr. Luedtke cites (Exs. 16 and 17)

merely explain what a safety-catch linker is—they do not discuss switching to a safety-catch linker midstream during oligomer synthesis. Respectfully, had a skilled artisan wished to use the safety-catch linker, she would have used it from the beginning of the synthesis method as described in those references.

31. Dr. Luedtke states that these hypothetical steps must be covered by the claims, because otherwise one "would avoid infringement." Luedtke Decl. ¶¶58, 59, 61. It is unclear how this is relevant to understanding the disputed phrase, and Dr. Luedtke does not provide an explanation. *See id.* In my view, both the claims and the specification support Sarepta's construction of step e) (that specifies the source of Compound 3 as step d)). Dr. Luedtke has not identified any reason that changes my opinion.

## 2. A Skilled Artisan Would Have Understood that Compound 3 Directly Reacts with a Deprotecting Agent

- 32. Dr. Luedtke agrees that chemical reactions involve different chemical compounds, some of which interact with each other to transform their chemical structures, while others facilitate the process without chemically reacting. *See* Luedtke Decl. ¶38; Pentelute Decl. ¶61. As previously explained, Compound 3 and a deprotecting agent fall in the former category. Sarepta's construction reflects this relationship, i.e., that they directly react with each other to chemically transform Compound 3 to Compound 4. Pentelute Decl. ¶60–63.
- 33. Both the claims and the specification support Sarepta's construction. First, the language of step e) specifies a direct reaction, "reacting said Compound 3 with a deprotecting agent." Ex. 2 at step e) of claims 1 and 6 (emphasis added). Step e) does not refer to any intermediate, indirect reactions. Further, as reproduced below, Step e) disclosed in the specification (corresponding to Step C in the specification) also instructs a skilled artisan to remove the protective group from Compound 3 (corresponding to Compound (VII) in the

specification) "using a deprotecting agent" without referencing any other reagents or indirect steps. *Id.* at 22:7–67. A skilled artisan reading step e) would have understood that Compound 3 and the deprotecting agent react directly.

- Or. Luedtke does not provide any evidence regarding the direct reaction between Compound 3 and the deprotecting agent of step e). *See* Luedtke Decl. ¶¶37–49. Instead, he focuses on step b) (i.e., *not* step e)), which is directed to "reacting said Compound 1" (i.e., *not* said Compound 3 as in step e)) "with an acid" (i.e., *not* a deprotecting agent as in step e)) to form Compound 2 (i.e., *not* Compound 4 as in step e)). Based on this discussion, he suggests that my interpretation of step e) is incorrect because the claim terms involve similar language. *Id.* ¶¶41–49.
- 35. Respectfully, I again disagree. To the contrary, Dr. Luedtke's analysis of step b) confirms that Sarepta's construction is correct. With respect to step b), Dr. Luedtke identifies three criticisms of Sarepta's construction: (1) an acid "can be used in the form of a dilution"; (2) "after the acid reaction, a second neutralization reaction may be required"; and (3) step b) could involve "multi-step and indirect reactions." *Id.* ¶¶43–48. As discussed in detail below, *none* of his examples changes the fundamental, *direct* nature of the reaction between Compound 1 and acid in step b), let alone the *direct* reaction between Compound 3 and a deprotecting agent in step e).

36. <u>Step b</u>): Step b) recites "reacting said Compound 1 with an acid to form Compound 2." As illustrated in **Figure 1** below, the acid (represented by H<sup>+</sup> in green) reacts with Compound 1 directly and causes the chemical transformation of Compound 1 to Compound 2. A skilled artisan reading step b) would have also understood that Compound 1 and the acid react *directly*, not indirectly.

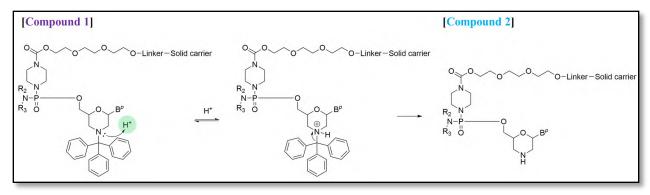


Figure 1. The Direct Reaction Between Compound 1 and an Acid in Step b)

- 37. <u>Dilution</u>: Similarly, using an acid in the form of a dilution does not change the direct reaction that occurs between Compound 1 and the acid. As the word suggests, an acid in the form of a dilution means that an acid that is diluted in a solution, for example, water (referred to as a diluent). The presence or absence of diluents or solvents, which do not participate in the chemical transformation of Compound 1 to Compound 2, does not change the *direct* reaction between Compound 1 and the acid depicted in **Figure 1**. To the extent that Dr. Luedtke is implying that Sarepta's construction excludes the presence of diluents or solvents, it does not, as previously explained. Pentelute Decl. ¶61, 64; *see also id*. ¶¶53–58.
- 38. <u>Second neutralization reaction</u>: Dr. Luedtke's "second neutralization reaction" is similarly irrelevant. In this context, neutralization refers to the process of eliminating any remaining acid with a base after the primary chemical reaction is completed. *See* Ex. 2 at 16:57–62. As shown in **Figure 2** below, performing a neutralization reaction (in the pink boxes) after

reacting an acid with Compound 1 does not change the fact that the acid and Compound 1 already reacted directly to form Compound 2. Further, as I previously discussed, Sarepta's construction does not exclude additional, non-transformative reactions such as neutralizations from the claims. See Pentelute Decl. ¶\$53–58.

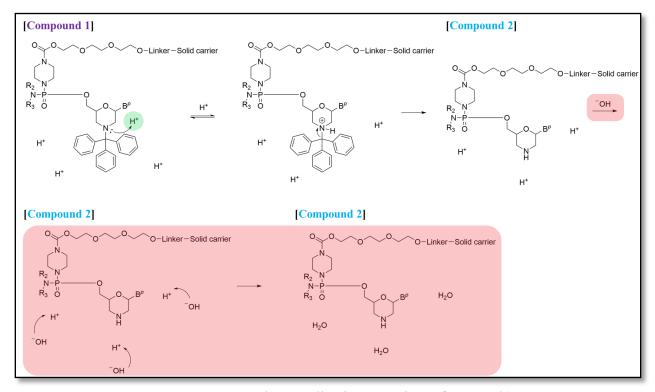


Figure 2. A Second Neutralization Reaction After Step b)

- 39. <u>Multi-step and indirect reactions</u>: Turning to step b) specifically and using trifluoroacetic acid as an example, Dr. Luedtke argues that step b) could involve "multi-step and indirect reactions" to form Compound 2. His characterization of step b) is irrelevant and inaccurate.
- 40. First, regardless of whether there are some hypothetical "multi-step and indirect" reactions involved, the fact that an acid necessarily reacts with Compound 1 *directly* does not change. Indeed, as corrected in **Figure 3** below, Dr. Luedtke's drawing of step b) entirely omits

the first, *direct* reaction between an acid and Compound 1 (in blue box) that must occur before the additional, hypothetical steps that he described.

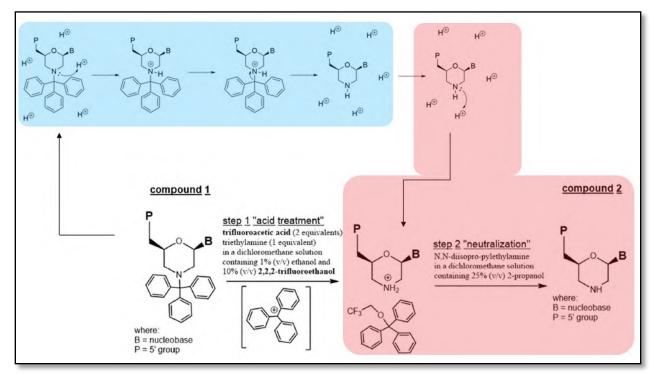


Figure 3. The Corrected Drawing of Dr. Luedtke's "Multi-Step" Depiction

- 41. Second, Dr. Luedtke's depiction of additional reactions that may occur after the completion of step b)—e.g., the addition of another proton (H<sup>+</sup>) to Compound 2 and the removal of that extra proton (in red box in **Figure 3** above)—is similarly irrelevant. He appears to assume that the acid reacting with Compound 1 will continue adding more protons even after the chemical transformation of Compound 1 to Compound 2 is complete. As an initial matter, the existence of any extra-protonated species is simply a hypothesis. Dr. Luedtke ignores the context of step b), which is part of a stepwise, multi-step solid-phase synthesis. Researchers would typically not separately isolate and evaluate the compounds formed at each step to test for protonation.
- 42. Dr. Luedtke also ignores the underlying chemistry. As depicted in his drawing, the reaction condition (2 equivalents of trifluoracetic acid (acid) and 1 equivalent of triethylamine (base)) would be expected to tightly regulate the appropriate acid level to properly protonate (and

not extra-protonate) the compound in the reaction. Further, he fails to account for the equilibrium achieved as the reaction progresses. Many Compound 2 molecules would remain as single-protonated because as the reaction progresses, acid molecules remaining in the reaction become scarce and any remaining acid molecules would preferentially interact with any remaining Compound 1 molecules in the reaction. Dr. Luedtke's artificial insertion of these additional steps ignores this basic aspect of the reaction, and regardless, that some Compound 2 molecules could theoretically be extra-protonated does not change that the acid reacts with Compound 1 directly.

- Third, the presence of "indirect reactions"—e.g., that as the acid reaction progresses, 2,2,2-trifluoroethanol could "indirectly drive the reaction forward"—is irrelevant. Luedtke Decl. ¶47. 2,2,2-trifluoroethanol does *not* react with Compound 1 directly, and indeed, Dr. Luedtke's drawing does not show any direct interaction between 2,2,2-trifluoroethanol and Compound 1. *See id.* ¶46. Rather, 2,2,2-trifluoroethanol facilitates the chemical transformation of Compound 1 by eliminating free trityl groups generated by the *direct* reaction between the acid and Compound 1. The presence of these additional indirect reactions does not change the direct nature of the reaction between the acid and Compound 1. In fact, it was known that 2,2,2-trifluoroethanol is not required for this reaction to proceed. Ex. 19 (MacCoss 1978) at 207 (using 90% aqueous 2,2,2-trifluoracetic acid without 2,2,2-trifluoroethanol to remove the trityl group from nucleosides). As I previously explained, Sarepta's construction does not exclude these indirect reactions. Pentelute Decl. ¶61, 64; *see also id.* ¶53–58.
- 44. <u>Step e)</u>: As illustrated above, none of these additional "multi-step and/or indirect reactions" change the fundamental, direct reaction between Compound 1 and the acid in step b). The same is true with step e). Dr. Luedtke does not identify similar "multi-step and/or indirect reactions" that could occur in the context of step e). But even if such reactions could occur, they

would not change the fact that Compound 3 and the deprotecting agent in step e) react directly, not indirectly.

### 3. NS's Construction Is Inconsistent with a Skilled Artisan's Understanding

- 45. In his declaration, Dr. Luedtke addresses NS's construction in a single paragraph, summarily asserting that a skilled artisan's understanding of step e) would be consistent with NS's construction. Luedtke Decl. ¶35. He provides no reasoning or explanation to support his conclusion. *See id.* Regardless, I maintain that NS's construction is flawed for several reasons. Pentelute Decl. ¶65–68.
- 46. First, NS's construction does not specify that Compound 3 used in step e) is from step d), and Dr. Luedtke in essence concedes that this omission would allow using "a Compound 3" from "any" source. Luedtke Decl. ¶54. Such a broad interpretation, as discussed above, is inconsistent with the claim language that expressly requires using "said Compound 3" from the prior step. It is also inconsistent with the teaching of the specification, which identifies step d) as the sole source of Compound 3. *See supra* ¶¶23–26.
- 47. Second, NS's construction does not specify that Compound 3 reacts with a deprotecting agent directly, expanding step e) to cover other, indirect reactions unsupported by the claims and the specification. For example, under NS's construction, step e) would allow converting Compound 3 to an unspecified compound, and then reacting that compound with a deprotecting agent. Neither the claims nor the specification contemplates this approach. *See supra* ¶¶32–44.
- 48. Finally, NS's construction as written states that the reaction is conducted "in order to form Compound 4." In other words, it appears to characterize the formation of Compound 4 as a "goal" rather than as a product that must be obtained. Dr. Luedtke acknowledges that replacing

"in order to form Compound 4" with "which results in Compound 4" (i.e., the phrase in Sarepta's construction) would be consistent with a skilled artisan's understanding of step e). Luedtke Decl. ¶35. I agree with Dr. Luedtke that Compound 4 must result from the reaction of step e), as is clear from Sarepta's construction.

B. Step f): "reacting Compound 4 with an acid to form said oligomer" or "reacting said Compound 4 with an acid to form said PMO"

Term	NS's Position	Sarepta's Position
"f) reacting	Plain and ordinary meaning—i.e.,	Plain and ordinary meaning, i.e.,
Compound 4 with	chemically reacting Compound 4	chemically reacting an acid
an acid to form said	with an acid, in order to form the	directly with Compound 4 of step
oligomer"	oligomer [or the PMO]	e), which results in the oligomer or
		the PMO.
"f) reacting said		
Compound 4 with		Step f) must occur after step e).
an acid to form said		
PMO"		

- 1. A Skilled Artisan Would Have Understood that the Claimed Steps, Including Step f), Must Be Performed in the Order Written
  - a. Dr. Luedtke Bases His Opinions on a Limitation Not in the Claims
- 49. Dr. Luedtke's positions regarding step f) are identical to those regarding step e), namely that "said Compound 4" or "Compound 4" in step f) "refers to a Compound 4 having the same previously defined structure and does not require Compound 4 to come from any specific final reaction." Luedtke Decl. ¶72–81.
- 50. I disagree for the same reasons discussed above. See supra ¶10–16. Dr. Luedtke's interpretation converts "said Compound 4" (or "Compound 4") to "a Compound 4" from "any" source. But this is inconsistent with both the claims and the specification. Step f) of claim 6 requires using "said Compound 4" from the previous step, not "a Compound 4" obtained from a different source. See Ex. 2 at step f) of claim 6. Step f) of claim 1 also does not mention using Compound 4 from any other source. See id. at step f) of claim 1. Further, the specification

expressly instructs a skilled artisan to use Compound 4 "produced in" the previous step, not some other, undefined step. *Id.* at 23:1–57 ("Compound (IX) *produced in* step C"). The specification does not discuss any other method for obtaining Compound 4. By changing the claim term "said Compound 4" or "Compound 4" to "a Compound 4," Dr. Luedtke adds a limitation that does not exist in the claims.

51. Dr. Luedtke's reasoning—that "said Compound 4" refers to the previously defined structure only—is similarly flawed, for the reasons discussed above. Luedtke Decl. ¶75; see supra ¶¶10–16. A shown below, the text of step e) explains that "Compound 4" is formed by reacting said Compound 3 with a deprotecting agent and the associated drawing depicts a defined chemical formula for the product, Compound 4:

A skilled artisan would have concluded from the express claim language and chemical structure that the claim term "said Compound 4" or "Compound 4" in step f) incorporates both aspects of Compound 4, i.e., its chemical structure and the underlying reaction used to make it.

### b. Dr. Luedtke's Hypothetical Reactions Inserted Between Steps e) and f) Are Irrelevant and Undesirable

- 52. Similar to step e), Dr. Luedtke states that certain "unrecited steps could be performed between steps e) and f) that could *alter* the result of step e)." Luedtke Decl. ¶77 (emphasis added). Citing Ex. 18, he argues that reactions such as a "transient silylation with TMS [an abbreviation of a compound known as trimethylchlorosilane]" could be performed between steps e) and f). § Id. ¶78. I disagree.
- 53. The plain language of the claims prohibits additional steps that alter the chemical structures of the recited Compounds. Step f) expressly requires using "said Compound 4" or "Compound 4"—the Compound 4 made in step f). Nothing in the claims or specification suggests performing additional, unrecited reactions that chemically transform Compound 4 from step e) to another, undefined compound. Indeed, the only sequence of reactions that the specification discloses includes step e) immediately followed by step f). *See* Pentelute Decl. ¶46–51; Ex. 2 at 22:7–67 (Step C: "In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX)."); *id.* at 23:1–57 (Step D: "PMO (I) is produced by reacting Compound (IX) *produced in Step C* with an acid."). A skilled artisan would not have understood that reactions "alter[ing] the result of step e)" between steps e) and f) (Luedtke Decl. ¶77) are permitted.

<sup>&</sup>lt;sup>3</sup> Dr. Luedtke again asserts that steps e) and f) are "polishing or finishing steps" "dependent on the purification methods available to" a skilled artisan. Luedtke Decl. ¶80. I have addressed his inaccurate characterization of these steps above. *See supra* ¶¶21–22.

54. The "transient silylation with TMS" Dr. Luedtke identified does not support his position. *See* Luedtke Decl. ¶78. As explained in Ex. 18 and further illustrated below, this transient silylation reaction aims to temporarily protect the *free* (unprotected) hydroxyl groups of a *sugar ring* of deoxynucleosides ("OH" highlighted in red). *See* Ex. 18 at Abstract. In contrast, the morpholine rings of Compound 4 do *not* have any free hydroxyl groups. Moreover, compared to individual deoxynucleosides, Compound 4 is a much larger molecule, containing numerous chemically reactive sites. Adding a chemically reactive reagent like TMS to Compound 4 can significantly alter the impurity profile and other aspects of the compound. A skilled artisan would not have concluded that this type of hypothetical reactions is desirable, let alone permitted.

55. As with step e), Dr. Luedtke again states that these hypothetical steps must be covered by the claims, because otherwise one "would avoid infringement." Luedtke Decl. ¶¶78, 79. It is unclear how this is relevant to understanding the disputed phrase, and Dr. Luedtke provides no explanation. *See id.* In my view, both the claims and the specification support Sarepta's construction of step f) (that specifies the source of Compound 4 as step e)). Dr. Luedtke has not identified any reason that changes my opinion.

### 2. A Skilled Artisan Would Have Understood that Compound 4 Directly Reacts with an Acid

- 56. Similar to step e), Dr. Luedtke largely relies on step b) and contends that because step b) can be a multi-step reaction, step f) must also encompass multi-step reactions. Based on this discussion, he suggests that my interpretation of step f) is incorrect because the claim terms involve similar language. Luedtke Decl. ¶¶66–71. I again disagree.
- 57. Similar to step e), Dr. Luedtke offers no opinion about the *direct* nature of the reaction between Compound 4 and an acid. *See id.* Regardless, a skilled artisan reading step f) would have understood that Compound 4 and an acid react directly. First, the language of step f) specifies a direct reaction, "*reacting* [said] Compound 4 *with* an acid." Ex. 2 at step f) of claims 1 and 6. Step f) does not refer to any intermediate, indirect reactions between Compound 4 and the acid. Further, as reproduced below, Step f) disclosed in the specification (corresponding to Step D in the specification) also instructs a skilled artisan to react Compound 4 (corresponding to Compound (IX) in the specification) *with* an acid without referencing any other reagents or indirect steps. *Id.* at 23:1–57. A skilled artisan reading step f) would have understood that Compound 4 and the acid react directly.

58. Further, as discussed above, Dr. Luedtke's characterization of step b) as a "multi-step" reaction is incorrect and irrelevant. *See supra* ¶¶35–43. The same applies to his

characterization of step f). While he speculates that "an intermediate compound containing  $H_2^+$  in place of the trityl group of Compound 4" may be formed during the reaction of step f) (Luedtke Decl. ¶69), he ignores that: (1) even if this could occur, many Compound 4 molecules would not be extra-protonated because the reaction will reach the equilibrium; and (2) regardless, Compound 4 reacts with an acid directly to form a PMO.

# 3. NS's Construction Is Inconsistent with a Skilled Artisan's Understanding

- 59. As with step e) above, Dr. Luedtke addresses NS's construction in a single paragraph, summarily asserting that a skilled artisan's understanding of step f) would be consistent with NS's construction. Luedtke Decl. ¶64. He provides no reasoning or explanation to support his conclusion. *See id.* I maintain that NS's construction is flawed for several reasons. Pentelute Decl. ¶¶82–86.
- 60. First, NS's construction does not specify that Compound 4 used in step f) is from step e), and Dr. Luedtke in essence concedes that this omission would allow using "a Compound 4" from "any" source. Luedtke Decl. ¶76. Such a broad interpretation, as discussed above, is inconsistent with the claim language that expressly requires using "said Compound 4" or "Compound 4" from the prior step. It is also inconsistent with the teaching of the specification, which identifies step e) as the sole source of Compound 4. *See supra* ¶¶49–51.
- 61. Second, NS's construction does not specify that Compound 4 reacts with an acid directly, expanding step f) to cover other, indirect steps unsupported by the claims and the specification. For example, under NS's construction, step f) would allow converting Compound 4 to an unspecified compound, and then reacting that compound with an acid. Neither the claims nor the specification contemplates this approach. *See supra* ¶56–58.

62. Finally, NS's construction as written states that the reaction is conducted "in order to form the oligomer [or the PMO]." In other words, it appears to characterize the formation of the oligomer and the PMO as a "goal" rather than as a product that must be obtained. Dr. Luedtke acknowledges that replacing "in order to form the oligomer [or the PMO]" with "which results in the oligomer [or the PMO]" (i.e., the phrase in Sarepta's construction) would be consistent with a skilled artisan's understanding of step f). Luedtke Decl. ¶64. I agree with Dr. Luedtke that the oligomer (claim 1) or PMO (claim 6) must result from the reaction of step f), as is clear from Sarepta's construction.

### Case 1:21-cv-01015-JLH Document 174-2 Filed 03/20/23 Page 316 of 318 PageID #: 7417

I declare under penalty of perjury that all statements made herein of my knowledge are true, and that all statements made herein on information and belief are believed to be true.

Date: March 13, 2023

Bradley L. Pentelute, Ph.D.

# **Appendix C**

### **List of Materials Considered**

Exhibit Number	Description
2	U.S. Patent No. 10,683,322
16	Flickinger et al., "Spatial Photorelease of Oligonucleotides, Using a Safety-Catch
	Photolabile Linker" Organic Letters, Vol. 8, No. 11 2357-2360 (2006)
17	Weichelt, F., "Topic: Safety-Catch Linker (SCAL)
18	Ti et al., "Transient Protection: Efficient One-Flask Syntheses of Protected
	Deoxynucleosides," J. Am. Chem. Soc., 104, 1316-1319 (1982)
19	MacCoss et al., "Facile detritylation of nucleoside derivatives by using
	trifluoracetic acid," Carbohydrate Res 60(1): 206-209 (1978) ("MacCoss 1978")